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GENETISCHE UNTERSUCHUNGEN AM BAKTERIOPHAGEN ΦX 174

I. AUFBAU EINES SELEKTIVEN SYSTEMS UND NACHWEIS GENETISCHER REKOMBINATION*

Von

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Mit 7 Textabbildungen

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Einleitung

TESSMAN (1959) und SINSHEIMER (1959) entdeckten, daß die DNS des Phagen ΦX 174 einsträngig ist. Dieser Phage ist um ein Vielfaches kleiner als die bekannten Phagen der T-Serie. Seine Größe ist vergleichbar der von kleinen RNS-Viren. Die DNS von ΦX hat — ähnlich wie die RNS solcher Viren — ein Molekulargewicht von etwa $2 \cdot 10^6$, was einer Kette von rund 6000 Nukleotiden entspricht.

Außer ΦX 174 ist noch ein weiterer Mikrophage, S 13, bekannt, der mit ΦX 174 serologisch nahe verwandt ist. Beide Phagen lassen sich auf den gleichen Wirtsbakterien *Shigella paradysenteria*, *Salmonella typhimurium*-, *E. coli C*-Stämmen vermehren und unterscheiden sich nur in ihren Ionenansprüchen bei der Vermehrung, ZAHLER (1957).

Genetische Untersuchungen an einem solchen Phagen mit einsträngiger DNS erschienen vom Standpunkt der Virusforschung im Hinblick auf pflanzliche und tierische Viren ähnlicher Größe, vom Standpunkt der molekularen Genetik aus wegen der außergewöhnlichen DNS dieser Phagen, für wünschenswert. Genetische Rekombination in Viren mit RNS sind bisher nur in einigen Fällen berichtet worden, HIRST (1959), WATSON (1960). Obwohl die technischen Schwierigkeiten bei Kreuzungen von pflanzlichen und tierischen Viren weitaus größer sind als bei Phagen, konnte genetische Rekombination bei ΦX 174 bisher nicht festgestellt werden. Lediglich bei S 13 gelang es TESSMAN (1959), nach UV-Stimulation Rekombinanten in einer Häufigkeit 0,1% aufzufinden. TESSMAN vermutet, daß das seltene Auftreten von Rekombinanten auf die geringe DNS-Menge des Phagen zurückzuführen sei. Die Voraussetzung für eine Untersuchung war also die Schaffung eines Systems zur selektiven Erfassung von Rekombinanten. Hierfür kam lediglich eine negative hostrange-Eigenschaft in Betracht, d.h. es mußten Phagen-Mutanten gewonnen werden, die im Gegensatz zu ihrem Ausgangstyp nicht mehr in der Lage waren, einen bestimmten Bakterienstamm anzugreifen. Die Kreuzung solcher Mutanten könnte durch Rekombination zum Ausgangstyp zurückführen, der auch in kleinsten Anteilen erfaßt würde.

Dieses Prinzip eines „negativen host-range-Systems" wurde zuerst von STREISINGER (1956) für Wirtsbereich-Mutanten des Phagen T 2 benutzt.

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Material

Bakterien. *E. coli C* und dessen Mutanten C_a, C_{ab}, C_{abc} als Wirtsbakterien; C_{abc} als selektives Bakterium für Rekombinanten. (Bemerkung: Der Stamm C_a ist leider bereits zu Beginn der Untersuchungen wieder eingegangen.)

Phagen. ΦX 174 Wildtyp und verschiedene Wirtsbereichmutanten.

Medium (Wachstumsmedium für Bakterien, Lysate usw.). Peptonbrühe = 10,0 g Pepton (Merck) auf 1000 ml Wasser, 5,0 g NaCl, 1,0 ml einer 1 M-$MgSO_4$-Lösung.

Platten. Farbplatten nach BRESCH und TRAUTNER (1956).

$NaNO_2$-Inaktivierungsmedium. 1 M-$NaNO_2$-Lösung, Na-Azetatlösung pH 4,7 (1 M-CH_3COOH + 1 M-CH_3COONa im Verhältnis 1:1 gemischt).

Versuchsergebnisse

I. Aufbau des selektiven Systems

a) Gewinnung der Ausgangsstämme. *E. coli C* wurde mit einem Überschuß von ΦX 174$^+$ plattiert. Die wenigen Bakterienkolonien, die nach 16stündigem Bebrüten der Platte bei 37° C gewachsen waren, wurden abgeimpft und auf Resistenz gegen ΦX 174$^+$ geprüft. Eine solche Bakterienmutante, auf der ΦX 174$^+$ keine Löcher mehr bilden konnte[1], wurde C_a genannt und bei 37° C in Peptonbrühe auf etwa $2 \cdot 10^8$/ml vermehrt. Mit diesen Bakterien wurden dann etwa 10^9 ΦX 174$^+$ plattiert, die auf der Bakterienmutante C_a zu 4—6 Löchern führten, die durch mutierte Phagenpartikel zu erklären sind.

Nach Isolierung einer solchen Phagenmutante (*Ha*) wurde diese auf C^+ vermehrt (der Titer des *Ha*-Lysates war auf C^+ bzw. C_a gleich) und nunmehr im Überschuß auf C_a plattiert. So konnten gegen ΦX *Ha* resistente Mutanten von C_a erhalten werden. Eine (C_{ab}) wurde mit 10^9 *Ha*-Phagen plattiert und so die Phagenmutante ΦX *HaHb* isoliert. In gleicher Weise wurde in einem dritten Schritt die Bakterienmutante C_{abc} und eine zugehörige Phagenmutante ΦX *HaHbHc*

Bakterien	Phagen
C^+	ΦX$^+$
↓	↓
C_a	ΦX *Ha*
↓	↓
C_{ab}	ΦX *HaHb*
↓	↓
C_{abc}	ΦX *HaHbHc*

Schema

Tabelle 1. *Wirtsbereich der in drei Schritten isolierten Mutanten von ΦX 174$^+$*

E. coli	C^+	C_a	C_{ab}	C_{abc}
ΦX$^+$	+	—	—	—
ΦX *Ha*	+	+	—	—
ΦX *HaHb*	+	+	+	—
ΦX *HaHbHc*	+	+	+	+

+ bildet Löcher, — bildet keine Löcher.

gewonnen (s. Schema und Tabelle 1). Versuche, in einem vierten Schritt, auf Mutanten von C_{abc}, also C_{abcd}-Mutanten, eine *HaHbHcHd*-Mutante zu finden, sind bisher ohne Erfolg geblieben.

b) Negative Wirtsbereich-Mutanten aus ΦX *HaHbHc*. Mit dem Ziel negative Wirtsbereich-Mutanten von *HaHbHc* zu erhalten, wurden Phagen mit Nitrit als Mutagen behandelt (SCHUSTER und SCHRAMM 1958, MUNDRY und GIERER 1958).

In 0,2 ml eines frisch angesetzten Gemisches von Natriumazetat- und Natriumnitrit-Lösung im Verhältnis 1:1 wurden bei 25° C 0,1 ml Phagenlysat *HaHbHc* (Titer 10^{10}) gegeben.

[1] Im weiteren Verlauf dieser Arbeit soll ein Bakterienstamm als „resistent" bezeichnet werden, wenn der betreffende Phage keine Löcher hervorruft. Offen bleibt dabei, ob dieser Phage unter Umständen noch an die Bakterien adsorbiert wird, ja sogar ob eine geringe Vermehrung der Phagen möglich ist.

Unter diesen Versuchsbedingungen wurde das *HaHbHc*-Lysat in 20—25 min von 10^{10} auf 10^6—10^5 inaktiviert.

Nach 25 min wurde das Reaktionsgemisch 1:1000 in Peptonbrühe verdünnt und mit einer Bakterienmischung $C^+ + C_{abc}$ (Verhältnis 1:2) aus Übernachtkulturen plattiert.

HaHbHc-Phagen bilden auf einem aus einer solchen Bakterienmischung entstandenen Rasen fast klare Löcher. Mutanten von *HaHbHc* konnten als trübe Löcher leicht erkannt und abgeimpft werden.

16 derartige Phagenmutanten wurden isoliert, gereinigt und aus ihnen Lysate hergestellt. Diese erhielten die Bezeichnung 1—16.

Nochmaliger Test zeigt Resistenz von C_{abc} gegen sämtliche 16 Stämme.

Somit liegen, ausgehend vom Phagenstamm *HaHbHc*, eine Reihe von 16 negativen Wirtsbereichmutanten vor. Zusammen mit dem Bakterienstamm C_{abc} bilden diese ein selektives System, das eventuelle, zwischen den Mutanten stattfindende Rekombination zum Ausgangstyp *HaHbHc* nachweisen würde.

II. Gruppen-Einteilung der Mutanten

Um zu prüfen, ob überhaupt Rekombinationen auftraten, wurde als Vorversuch ein Tropfentest durchgeführt.

Auf je eine Agar-Platte wurden 0,2 ml einer Bakterienmischung von $C^+ + C_{abc}$ (1:4) aus Übernachtkulturen ausgestrichen[1].

Nach etwa 15—20 min war die Bakterienlösung aufgetrocknet und die Platten konnten zum Auftropfen der Lysate benutzt werden. Je 0,2 ml der Lysate negativer Wirtsbereichmutanten von *HaHbHc* (mit einem Titer von $5 \cdot 10^8$) wurden in allen Kombinationen gemischt und 0,005 ml mit einer Mikropipette aufgetropft. Zur Kontrolle wurde jedes Lysat für sich allein in gleicher Konzentration getestet.

Die Kontroll-Spots waren nach 16stündigem Bebrüten der Platten bei 37° C trübe, während eine größere Anzahl von Misch-Spots völlig klar war oder innerhalb der etwa 1—1,5 cm großen Spots einige 2—3 mm große, klare Einzellöcher zeigten.

Tabelle 2. *Tropfentest (Spot-Test) von 18 Mutanten; Rekombinanten durch „+", keine Rekombinanten durch „—" dargestellt*

Mut.	*8*	*11*	*12*	*16*	*1*	*5*	*13*	*14*	*15*	*4*	*6*	*7*	*9*	*10*	*HaHb*	*Ha*	*3*	*2*
8	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	—
11	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	—
12	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	—
16	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+	+	—
1	+	+	+	+	—	—	—	—	—	+	+	+	+	+	+	—	—	+
5	+	+	+	+	—	—	—	—	—	+	+	+	+	+	+	—	—	+
13	+	+	+	+	—	—	—	—	—	+	+	+	+	+	+	—	—	+
14	+	+	+	+	—	—	—	—	—	+	+	+	+	+	+	—	—	+
15	+	+	+	+	—	—	—	—	—	+	+	+	+	+	+	—	—	+
4	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
6	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
7	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
9	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
10	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
HaHb	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
Ha	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	+	+	+	+	+	—	—	—	—	—	—	—	—	—

[1] Da mit Sicherheit eine Vermehrung der Rückmutanten nur in C^+-Zellen stattfindet, mußte für den Tropfentest ein Mischrasen aus $C^+ + C_{abc}$ gewählt werden. Eventuell gebildete *HaHbHc*-Rekombinanten würden dann durch Lysieren beider Rasen erkennbar werden.

Tabelle 2 zeigt die Kombinationsmöglichkeiten der 18 Mutanten, 1—16, *Ha* und *HaHb* im Tropfentest und das Ergebnis dieses Versuches, PFEIFER (1961).

Vernachlässigt man zunächst die Mutanten *Ha*, *2* und *3*, so lassen sich die übrigen Stämme im Tropfen-Versuch in drei Gruppen aufteilen:

Bei Tropfenkreuzungen zweier Stämme der gleichen Gruppe ist das Resultat stets negativ, bei solchen aus verschiedenen Gruppen immer positiv. Tabelle 3a gibt diese Gruppeneinteilung wieder.

Tabelle 3a. *Gruppeneinteilung der Mutanten*

8	*1*	*4*
11	*5*	*6*
12	*13*	*7*
16	*14*	*9*
	15	*10*
		HaHb

Zum Verständnis dieser Situation vergegenwärtige man sich nochmals die Entstehungsgeschichte der Mutanten:

der Wildphage	*+++*
mutierte zu	*Ha++*
weiter zu	*HaHb+*
und schließlich zu	*HaHbHc*

Zur Lochbildung auf C_{abc} ist in jedem Fall diese Genom-Konstellation: *HaHbHc* erforderlich. Der Verlust des Wirtsbereiches (Fähigkeit der Lochbildung auf C_{abc}) könnte eintreten durch eine Veränderung im locus *Ha* oder *Hb* oder *Hc* oder an einer vierten Stelle.

Kreuzt man die Mutante *Ha* mit einer der 16 „funktionellen Rückmutanten"[1]), so sollte ein Rekombinantentyp *HaHbHc* nur dann gebildet werden, wenn der betreffende Stamm sowohl den locus *Hb* als auch den locus *Hc* in unveränderter

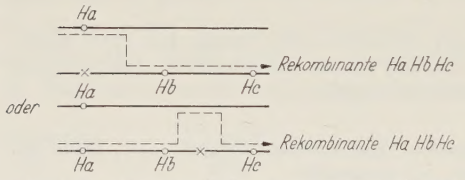


Abb. 1. Beispiele einer Rekombination zu *HaHbHc*. × funktionelle Rückmutation

Tabelle 3b. *Symbolik von Tabelle 3a*

Ha	*Hb*	*Hc*
8 Hb Hc	*Ha 1 Hc*	*Ha Hb 4*
11 Hb Hc	*Ha 5 Hc*	*Ha Hb 6*
12 Hb Hc	*Ha 13 Hc*	*Ha Hb 7*
16 Hb Hc	*Ha 14 Hc*	*Ha Hb 9*
	Ha 15 Hc	*Ha Hb 10*
		Ha Hb +

Form enthält. Die funktionelle Rückmutation muß also im locus *Ha* selbst oder an einem vierten Ort erfolgt sein (vgl. Abb. 1).

Da die funktionellen Rückmutanten sich in drei Gruppen einteilen lassen, liegt es nahe, diese Gruppen mit Rückmutationen in den drei loci *Ha*, *Hb* und *Hc* zu identifizieren. Die Gruppe, die mit dem Stamm *Ha* rekombiniert, sollte demnach im locus *Ha* rückmutiert sein (*Hb* und *Hc* blieben erhalten).

Auch der Stamm *HaHb* sollte danach mit den gleichen Stämmen wie *Ha* zu Rekombinanten *HaHbHc* führen, wie von Tabelle 2 bestätigt wird. Darüber hinaus sollte *HaHb* aber auch mit all den funktionellen Rückmutanten Rekombinanten *HaHbHc* liefern, die im locus *Hb* rückmutierten. Hierdurch kann eine zweite Gruppe als Rückmutanten in *Hb* identifiziert werden. Der dritten Gruppe müßten damit Rückmutationen im locus *Hc* zugeschrieben werden.

[1] Die Bezeichnung „Funktionelle Rückmutante" soll veranschaulichen, daß zwar in bezug auf die „Funktion" von *HaHbHc* (Lochbildung auf C_{abc}) eine „Rück"-Mutation erfolgte, daß aber andererseits im Einzelfall offenbleibt, ob tatsächlich ein bei der Hin-Mutation veränderter locus dabei wieder seine ursprüngliche Konfiguration angenommen hat.

In deutlicher Symbolik können die Stämme von Tabelle 3a unter dieser Annahme durch Tabelle 3b wiedergegeben werden.

Es sei darauf hingewiesen, daß die Ziffern zwar formal als „Allele" der betreffenden loci benutzt werden, daß aber andererseits eine Mutation an einem vierten Ort nicht auszuschließen ist. In einem solchen Fall würde dann formal dieser vierte Ort mit dem funktionell verbundenen locus zu einem „Allel" dieses locus zusammengefaßt werden (vgl. Abb. 2).

Unter der Bezeichnung *HaHb+* wurde auch der Stamm *HaHb* unter die funktionellen Rückmutanten aufgenommen. Die Mutante *Ha++* konnte weder mit den in *Hb* noch den in *Hc* funktionell rückmutierten Stämmen zum *HaHbHc*-Typ rekombinieren. Diese Eigenschaft, mit zwei der drei Gruppen nicht rekombinierbar zu sein, zeigen auch die Stämme 2 und 3. Es

Tabelle 3c. *Formale Symbolik sämtlicher Mutanten (s. Text)*

Ha	*Hb*	*Hc*
2 Hb 2	*Ha 3 3*	*2 Hb 2*
8 Hb Hc	*Ha 1 Hc*	*Ha 3 3*
11 Hb Hc	*Ha 5 Hc*	*Ha Hb 4*
12 Hb Hc	*Ha 13 Hc*	*Ha Hb 6*
16 Hb Hc	*Ha 14 Hc*	*Ha Hb 7*
	Ha 15 Hc	*Ha Hb 9*
	Ha + +	*Ha Hb 10*
		Ha Hb +
		Ha + +

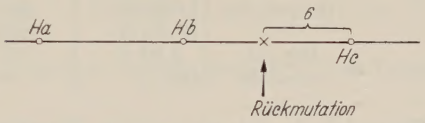


Abb. 2. Beispiel einer möglichen Genomkonfiguration und ihrer formalen Symbolik

ist daher plausibel anzunehmen, daß auch diese (zumindest funktionell) in zwei der drei loci rückmutierten. In formaler Schreibweise lassen sich daher diese Mutanten durch *2 Hb 2* bzw. *Ha 3 3* charakterisieren.

Trägt man diese Stämme und dazu die Mutante *Ha++* doppelt in die Tabelle (3c) ein, so gilt die Regel, daß jeder Mutantenstamm mit allen anderen zur Rekombinante *HaHbHc* führt, ausgenommen diejenigen Stämme, die zur gleichen Gruppe gehören.

III. Anordnung der loci Ha, Hb, Hc

Die bisher benutzte Reihenfolge bei der Schreibweise der loci *HaHbHc* entspricht deren Entstehungsgeschichte, nicht aber einer realen Anordnung. Um diese zu ermitteln, wurde die Genauigkeit des Tropfen-Test durch eine quantitativere Technik verbessert:

„Plattenkreuzungen". Lysate zweier Mutanten wurden im Verhältnis 1:1 gemischt und so verdünnt, daß der Gesamttiter $5 \cdot 10^8$ betrug. 0,25 ml der Lysatmischung wurden in 1,0 ml Bakterien $C^+ + C_{abc}$ (1:4) von Übernachtkulturen pipettiert und von dieser Phagen-Bakterienmischung sofort 0,25 ml auf einer Platte ausgestrichen. Die Platten wurden 16 Std bei 37° C bebrütet.

Mit dieser Technik werden nicht (wie sonst üblich) die einzelnen Rekombinanten gezählt, sondern die Wirtszellen, in denen Rekombination erfolgte. Überall da, wo der Tropfen-Test positiv ausgefallen war, zeigte sich auf den Platten eine verschieden große Anzahl einzelner, klarer Löcher von 2—3 mm Durchmesser (Tabelle 4).

In Übereinstimmung mit der Annahme, daß die zu Gruppen zusammengefaßten Stämme in gleichen loci rückmutiert sind, liefern die Kreuzungen

zwischen verschiedenen Gruppen — unabhängig davon, welche Stämme der Gruppen im Einzelfall benutzt werden — etwa gleiche Werte:

Gruppe *Ha* × Gruppe *Hb* : etwa 750
Gruppe *Ha* × Gruppe *Hc* : etwa 1700
Gruppe *Hb* × Gruppe *Hc* : etwa 1700

Tabelle 4. *Beispiele von „Plattenkreuzungen". Die Zahlen geben jeweils die Summe von auf zwei Platten gezählten Rekombinanten-Löcher verschiedener Kreuzungen wieder*

		Gruppe *Hb*		Gruppe *Ha*	
		Ha 1 Hc	*Ha 14 Hc*	*8 Hb Hc*	*11 Hb Hc*
Gruppe *Hc*	*Ha Hb 4*	1611	1752	1661	1665
	Ha Hb 6	1742	1708	1752	1751
	Ha Hb 7	1621	1693	1835	1665
	Ha Hb 9	1713	1726	1681	1628
	Ha Hb 10	1515	1638	1592	1548
Gruppe *Ha*	*8 Hb Hc*	850	688	Selbstung	
	11 Hb Hc	747	895		
	12 Hb Hc	810	704		
Doppel-mutante	*2 Hb 2*	572	588	0	0
	Ha 3 3	0	0	90	101

Formal ließe sich eine solche Situation durch eine Dreieckskonfiguration oder durch zwei Kopplungsgruppen beschreiben (Abb. 3).

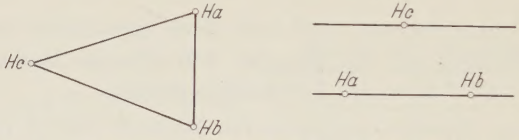


Abb. 3. Anordnungsmöglichkeiten der drei Mutantengruppen

Will man bei konventionellen Vorstellungen bleiben, d.h. nur *eine* eindimensionale Genom-Struktur annehmen, die auf Grund des DNS-Moleküls auch die plausibelste Deutung wäre, so könnten die Zahlenverhältnisse durch eine Sättigung der Rekombinationswahrscheinlichkeiten verstanden werden [BRESCH (1959)].

Damit ergäbe sich eine Reihenfolge der loci:

—— *Ha* —— *Hb* ———— *Hc* —— oder —— *Hb* —— *Ha* ———— *Hc* ——

Eine andere Deutungsmöglichkeit wäre eine ringförmige Genom-Struktur.

Inter-Gruppen-Kreuzungen bieten also keine Möglichkeit, eine endgültige Lokalisation der beteiligten Mutationsorte vorzunehmen.

IV. Intra-Gruppen-Kreuzungen

Auch innerhalb einer Gruppe zeigen die funktionellen Rückmutanten zum Teil erhebliche phänotypische Unterschiede bei der Lochbildung auf einem Mischrasen von $C^+ + C_{ab}$, wodurch eine genetische Identität ausgeschlossen wird.

Die Gruppe *Ha* ist in Tabelle 5a im einzelnen beschrieben.

Es schien daher möglich, daß auch innerhalb einer Gruppe Rekombination nachzuweisen wäre. Zu diesem Zweck wurden Kreuzungen mit folgender Technik angelegt:

0,2 ml einer Lysatmischung (Gesamttiter 1,0—1,5 · 10^9/ml im Verhältnis 1:1 der Parentaltypen) wurden in einem Zentrifugenröhrchen mit 0,1 ml einer frischwachsenden C^+-Kultur (Titer etwa 2,0 · 10^8/ml) bei 37° C zur Adsorption gebracht.

Nach 5 min wurde die Adsorption durch eine 1:50-Verdünnung des Phagen-Bakterien-gemisches mit eiskalter Peptonbrühe gestoppt und die freien Phagen von den Phagen-Bakterien-Komplexen durch $3^1/_2$ min Zentrifugieren getrennt.

Nach Dekantieren des Überstandes wurde das Sediment mit eiskalter Peptonbrühe aufgenommen und durch nochmaliges Zentrifugieren die restlichen freien Phagen von den Komplexen getrennt. Anschließend wurden die Komplexe mit Peptonbrühe 1:100 verdünnt und zum Lysieren für 45 min in einem Wasserbad bei 37° C aufbewahrt. Danach wurden in geeigneten Verdünnungen die Rekombinanten auf C_{abc} und die Parentalnachkommen auf C^+ bzw. $C^+ + C_{ab}$ bestimmt.

Selbstungen wurden wie die Kreuzungen durchgeführt, nur daß anstelle eines Mischlysates das Lysat eines Parentaltyps allein zur Adsorption an C^+ gebracht wurde.

Tabelle 5a. *Lochmorphologische Unterschiede auf $C^+ + C_{ab}$-Mischrasen, der funktionellen Rückmutanten aus der Gruppe Ha*

Mutantengruppe *Ha*				
Mutante	Rand		Lochzentrum	
	schmal, blau	rand-los	klar	trübe
16 Hb Hc	+		+	
8 Hb Hc	+			+
12 Hb Hc	+			+
11 Hb Hc		+	+	

Die Ergebnisse solcher Kreuzungen innerhalb der *Ha*-Gruppe sind in Tabelle 5b zusammengestellt.

Unter Berücksichtigung der Selbstungswerte kann man erkennen, daß die phänotypisch gleichen Mutanten 8 und 12 in Kreuzungen keine Rekombinanten liefern und daher genotypisch identisch sein können.

Zur genaueren Auswertung muß das unterschiedliche Parentaltypenverhältnis in der Nachkommenschaft beachtet werden. Dieses Verhältnis ist für jede einzelne Kreuzung (auch bei Inter-Gruppen-Kreuzungen) unter gleichen Bedingungen konstant. Fast in allen Fällen ist aber die Nachkommenschaft des einen Kreuzungspartners um ein Mehrfaches größer als die des anderen, obwohl die beiden Elterntypen im Adsorptions-Röhrchen in gleicher Konzentration vorlagen.

Tabelle 5b. *Rekombinantenhäufigkeit HaHbHc von Intra-Gruppen-Kreuzungen der Mutantengruppe Ha und Nachkommenverhältnisse der Parentaltypen*

Kreuzung	Nachkommenverhältnis	Rekombinanten: *HaHbHc*	
		unkorrigiert	korrigiert
		× 10^{-6}	
16 Hb Hc × *11 Hb Hc*	2,7:1,0	46	60
16 Hb Hc × *8 Hb Hc*	10,0:1,0	25	(75)
11 Hb Hc × *8 Hb Hc*	1,7:1,0	27	30
8 Hb Hc × *12 Hb Hc*	nicht unterscheidbar	0,2	0,2
Selbstung			
16 Hb Hc		2	
8 Hb Hc		< 0,3	
11 Hb Hc		3	
12 Hb Hc		< 0,2	

Zur Zeit kann noch nicht entschieden werden, welche Ursachen hierfür vorliegen. Adsorptionsmessungen und Einzelwurfexperimente bei einigen Kreuzungen ergaben Unterschiede in der Schnelligkeit der Adsorption der Partner und/oder unterschiedliche Vermehrung in mischinfizierten Zellen.

Zum Vergleich von Rekombinations-Wahrscheinlichkeiten ist es nötig, diesen Nebeneffekt zu berücksichtigen und durch eine Korrektur möglichst zu eliminieren.

Obwohl noch keine Einzelheiten über den Rekombinationsprozeß bei einsträngigen Phagen bekannt sind, erschien es am sinnvollsten, in Analogie zu anderen Phagen eine paarweise Kooperation zwischen zufälligen vegetativen Partikeln anzunehmen. Dementsprechend sollte die Wahrscheinlichkeit einer Kooperation zwischen verschiedenen Partnern von 0,5, ihrem Wert bei einem 0,5:0,5 Elternverhältnis im Pool, kleiner werden um einen Faktor $2 \cdot (1 - x)/0{,}5$, wenn statt des 0,5:0,5 ein Elternverhältnis von $x : 1 - x$ vorliegt.

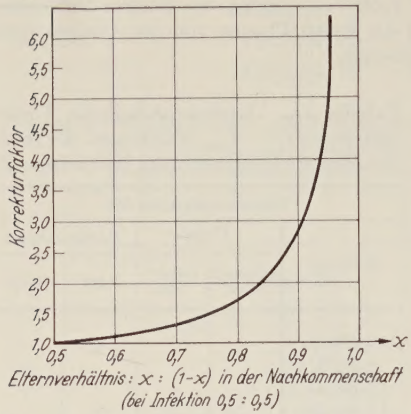


Abb. 4. Korrekturkurve der Rekombinantenwerte

Um auf die Rekombination bei symmetrischer Elternsituation zu normieren, müßte umgekehrt der gefundene Rekombinationsprozentsatz mit $0{,}5/2 \cdot (1 - x)$ multipliziert werden, wenn das betreffende Elternverhältnis während der gesamten Latenzperiode herrschen würde. Diese Bedingung ist erfüllt, wenn im wesentlichen unterschiedliche Adsorption für die Verschiebung des Elterntypenverhältnisses verantwortlich wäre.

Unter dieser Annahme ergibt sich die Korrekturkurve von Abb. 4. Diese kann jedoch nur als grobe Abschätzung einer Korrektur angesehen werden, da

1. das Elternverhältnis sich durch ungleiche Vermehrung erst im Laufe der Latenzperiode verschieben kann (Korrektur zu groß),

2. Das Verhältnis der Elterntypen in den Einzelzellen wesentlich von dem mittleren der Gesamtpopulation abweichen kann. Anzeichen hierfür liegen aus Einzel-Wurf-Untersuchungen vor (Korrektur zu klein).

Die so korrigierten Rekombinanten-Prozentsätze sind in den Tabellen 5b, 6b, 7b, 8, 9 und 10 ebenfalls aufgeführt.

Tabelle 6a. *Lochmorphologische Unterschiede auf $C^+ + C_{ab}$-Mischrasen der funktionellen Rückmutanten aus Hb*

Mutante	Mutantengruppe *Hb*			
	blauer Rand		Lochzentrum	
	schmal	sehr breit	klar	trübe
Ha 1 Hc	+			+
Ha 5 Hc	+			+
Ha 13 Hc	+			+
Ha 14 Hc	+			+
Ha 15 Hc		+	+	

Tabelle 6b. *Rekombinantenhäufigkeit HaHbHc von Intra-Gruppen-Kreuzungen der Mutantengruppe Hb und Nachkommenverhältnisse der Parentaltypen*

Kreuzung	Nachkommenverhältnis	Rekombinanten: *HaHbHc*	
		unkorrigiert	korrigiert
		$\times 10^{-6}$	
Ha 1 Hc × *Ha 14 Hc*	nicht unterscheidbar	0,2	0,2
Ha 1 Hc × *Ha 15 Hc*	1,0:5,0	0,3	0,3
Selbstung *Ha 1 Hc*		$< 0{,}3$	
Ha 14 Hc		$< 0{,}2$	
Ha 15 Hc		$< 0{,}5$	

Auch ohne Benutzung der korrigierten Werte ist jedoch aus dem Auftreten von Rekombinanten ersichtlich, daß die Rückmutationen im *Ha*-locus — wenigstens zum Teil — an verschiedenen Stellen ausgelöst wurden. Rekombination zwischen diesen ist jedoch zu selten, um im Tropfen-Test (vg. II.) erfaßt zu werden.

Analoge Resultate ergaben sich in den Mutantengruppen *Hb* und *Hc*, deren Einzelheiten aus der Tabelle 6 und 7 ersichtlich sind. In der Gruppe *Hb* wurde auch zwischen phänotypisch unterschiedlichen Mutanten keine Rekombination beobachtet.

Die Mutanten *Ha 5 Hc* und *Ha 13 Hc* wurden für Kreuzungen nicht verwendet, da in den Lysaten eine größere Anzahl phänotypisch verschiedener Typen auftraten.

V. Inter-Gruppen-Kreuzungen

Schließlich wurde versucht, durch normale Kreuzungstechnik genauere Werte als die aus Plattenkreuzungen auch für Inter-Gruppen-Kreuzungen zu erhalten. Hierbei wurde bestätigt, daß die Rekombinantenhäufigkeit zwischen den Gruppen *Ha* × *Hc* und *Hb* × *Hc* keine Unterschiede erkennen läßt und zwischen *Ha* × *Hb*, analog dem Ergebnis der Plattenkreuzungstechnik, geringere Werte aufweist (s. Tabelle 8).

VI. Kreuzungsanalysen mit Doppelmutanten

Außer acht gelassen wurden bisher Kreuzungen mit Partnern, die anscheinend Änderungen an zwei Stellen des *HaHbHc*-Typs tragen: *2 Hb 2*, *Ha 3 3* und *Ha* + +.

Tabelle 7a. *Lochmorphologische Unterschiede auf $C^+ + C_{ab}$-Mischrasen der funktionellen Rückmutanten aus der Gruppe Hc*

Mutantengruppe *Hc*					
Mutante	blauer Rand		randlos	Lochzentrum	
	schmal	breit		klar	trübe
Ha Hb +		+		+	
Ha Hb 4			+		+
Ha Hb 6			+		+
Ha Hb 7			+		+
Ha Hb 9			+		+
Ha Hb 10	+			+	

Tabelle 7b. *Rekombinantenhäufigkeit HaHbHc von Intra-Gruppen-Kreuzungen der Mutantengruppe Hc und Nachkommenverhältnisse der Parentaltypen*

Kreuzung	Nachkommenverhältnis	Rekombinanten: *HaHbHc*	
		unkorrigiert	korrigiert
		× 10^{-6}	
Ha Hb+ × *Ha Hb 6*	3,6:1,0	41	60
Ha Hb+ × *Ha Hb 10*	1,0:1,0	60	60
Ha Hb 6 × *Ha Hb 10*	1,0:3,0	8	10
Ha Hb 6 × *Ha Hb 7*	nicht unterscheidbar	0,1	0,1
Ha Hb 4 × *Ha Hb 9*	desgl.	0,2	0,2
Ha Hb 7 × *Ha Hb 9*	desgl.	0,1	0,1
Selbstung			
Ha Hb +		6	
Ha Hb 4		< 0,1	
Ha Hb 6		< 0,1	
Ha Hb 7		< 0,1	
Ha Hb 9		< 0,1	
Ha Hb 10		< 0,5	

Tabelle 8. *Beispiele der Rekombinantenhäufigkeiten HaHbHc aus Inter-Gruppen-Kreuzungen*

Inter-Gruppen-Kreuzung	Kreuzung	Nachkommenverhältnis	Rekombinanten: *HaHbHc*	
			unkorrigiert	korrigiert
			× 10^{-6}	
Ha × *Hb*	*8 Hb Hc* × *Ha 1 Hc*	1,7:1,0	290	319
	16 Hb Hc × *Ha 15 Hc*	1,7:1,0	340	370
	11 Hb Hc × *Ha 15 Hc*	1,0:1,5	280	294
	8 Hb Hc × *Ha 15 Hc*	1,0:4,0	120	187
Ha × *Hc*	*16 Hb Hc* × *Ha Hb 10*	nicht unterscheidbar	440	440
	11 Hb Hc × *Ha Hb 10*	1,0:1,0	410	410
	8 Hb Hc × *Ha Hb 7*	1,0:1,0	475	475
Hb × *Hc*	*Ha 15 Hc* × *Ha Hb 10*	1,5:1,0	455	478
	Ha 1 Hc × *Ha Hb 7*	1,0:1,7	475	525

Unter Berücksichtigung der Selbstungen konnte keine Rekombination bei den Kreuzungen beobachtet werden, die in Tabelle 9 aufgeführt sind. Offenbar liegen die Mutationen an denselben Stellen oder verlangen bei geringen Abständen mehrere Rekombinationen, so daß ein Anstieg von *HaHbHc* durch Rekombination gegenüber der Mutation nicht mehr gesichert werden kann.

Alle übrigen Kreuzungen mit diesen Doppelmutanten lassen einen deutlichen Zuwachs von *HaHbHc* aus Rekombination erkennen. Er ist aber in jedem untersuchten Fall kleiner als der aus gewöhnlichen Inter-Gruppen-Kreuzungen (siehe Tabelle 10).

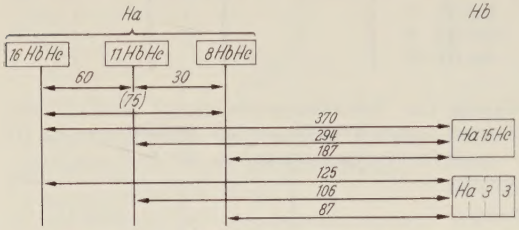


Abb. 5. Anordnung der Mutanten in „*Ha*". ▭ Einfachmutante, ▭▭ Doppelmutante

Tabelle 9. *Kreuzungen mit Doppelmutanten, die keine Rekombinanten HaHbHc hervorbringen*

Kreuzung	Anzahl von *HaHbHc* × 10^{-6}
Ha + + × Ha 3 3	< 0,5
Ha + + × 2 Hb 2	< 0,1
Ha + + × Ha 15 Hc	< 0,2
Ha + + × Ha Hb +	3
Selbstung	
Ha + +	< 0,1
Ha Hb +	6
Ha 3 3	< 0,8
2 Hb 2	< 0,1
Ha 15 Hc	< 0,5

Tabelle 10. *Kreuzungsergebnisse mit Doppelmutanten*

Kreuzung	Nachkommenverhältnis	Rekombinanten: *HaHbHc*	
		unkorrigiert	korrigiert
		× 10^{-6}	
2 Hb 2 × 8 Hb Hc	nicht unterscheidbar	8	8
2 Hb 2 × 11 Hb Hc	1,0:3,0	9	12
2Hb 2 × 16 Hb Hc	1,0:25,0(!)	7	*
Ha 3 3 × 8 Hb Hc	2,7:1,0	67	87
Ha 3 3 × 11 Hb Hc	1,7:1,0	95	106
Ha 3 3 × 16 Hb Hc	1,0:4,0	80	125
Ha + + × 11 Hb Hc	1,0:1,0	88	88
Ha 3 3 × Ha Hb 10	1,3:1,0	12	12
Ha 3 3 × Ha Hb 6	4,0:1,0	7	11
2 Hb 2 × Ha Hb 10	1,0:2,0	8	10
2 Hb 2 × Ha Hb 6	1,0:1,0	7	7
Ha + + × Ha Hb 6	3,0:1,0	9	12
Ha + + × Ha 14 Hc	1,6:1,0	2	2
2 Hb 2 × Ha 14 Hc	nicht unterscheidbar	110	110

* Korrektur erscheint sinnlos.

Diskussion

Wenn auch die verschiedenen Daten der Kreuzungsanalysen nicht ausreichen, um eine Reihenfolge der Mutantengruppen *Ha*, *Hb* und *Hc* festzulegen, so erlauben sie doch, wenigstens die Gruppen *Ha* und *Hb* in Beziehung zu setzen und hier innerhalb der Gruppe *Ha* eine Additivität der Mutationsabstände wahrscheinlich zu machen.

Abb. 5 veranschaulicht graphisch die Rekombinantenhäufigkeiten zwischen verschiedenen Mutanten (Werte den Tabellen 5, 8 und 10 entnommen).

Die Rekombinantenhäufigkeiten der Kreuzungen von *Ha 15 Hc* einerseits und *Ha 3 3* andererseits mit *16 Hb Hc*, *11 Hb Hc* und *8 Hb Hc* weisen eine Staffelung auf, die der der Intra-Gruppen-Kreuzungswerte von *16 Hb Hc*, *11 Hb Hc* und *8 Hb Hc* etwa entspricht und eine Mutantenanordnung $\frac{16\ \ 11\ \ 8}{Ha \qquad Hb}$ vermuten läßt.

Auffallend ist, daß die Kreuzungswerte von *Ha 15 Hc* mit *16 Hb Hc*, *11 Hb Hc* und *8 Hb Hc* um etwas mehr als die Hälfte höher sind als die der Doppelmutante *Ha 3 3* mit *16 Hb Hc*, *11 Hb Hc* und *8 Hb Hc*.

Dieser Befund läßt sich in zweifacher Weise deuten: einerseits durch die Existenz einer starken negativen Interferenz und andererseits durch zufallsgemäße Öffnung einer ringförmigen Kopplungsgruppe.

I. Starke negative Interferenz

Wie auch immer die Anordnung der loci sei, *Ha—Hb———Hc* oder *Hc———Ha—Hb*, die Wahrscheinlichkeiten einer Einfachrekombination und einer Doppelrekombination sind etwa gleicher Größe, wie aus Abb. 6 ersichtlich wird. Derartig häufiges Auftreten von Doppelrekombination wird als starke negative Interferenz bezeichnet. Das gleiche Phänomen zeigt sich auch in anderen Kreuzungen. Zeichnet man z. B. die möglichen Anordnungen der loci für die Kreuzungen

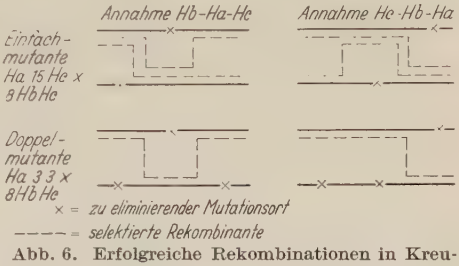


Abb. 6. Erfolgreiche Rekombinationen in Kreuzungen mit Einfach- und Doppelmutanten für zwei mögliche Anordnungen der loci *Ha*, *Hb* und *Hc*

Ha 14 Hc × *2 Hb 2*

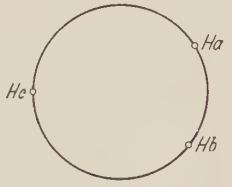
und

16 Hb Hc × *Ha 3 3* (Tabelle 10),

so erkennt man, daß in jedem Falle eine dieser beiden Kreuzungen Einfachrekombination, die andere Doppelrekombination verlangt. Da beide etwa zu gleichen Rekombinantenzahlen führen, ergibt sich auch hier eine etwa gleiche Häufigkeit für Doppel- und Einfachrekombination.

II. Ringförmige Genomstruktur

Eine andere Erklärung dieser Versuchsergebnisse wäre die Existenz einer ringförmigen Struktur:



Würde dieser Ring zur Replikation ebenso wahrscheinlich zwischen *Hc* und *Ha* wie zwischen *Hc* und *Hb* geöffnet, so würden die beiden bisher als alternativ angesehenen Möglichkeiten

Ha—Hb———Hc und *Hc———Ha—Hb*

in gleicher Häufigkeit auftreten. (Eine Öffnung zwischen *Ha* und *Hb* könnte selten oder überhaut nicht möglich sein.)

Unter dieser Annahme würde *Einfachrekombination* zur Erklärung aller Kreuzungsdaten ausreichen. Diese Betrachtungsweise ist schematisch in Abb. 7 dargestellt. Es ergibt sich, daß

a) Kreuzungen mit *Ha* × *Hc* und *Hb* × *Hc* gleiche und zugleich die größten Rekombinantenwerte liefern,

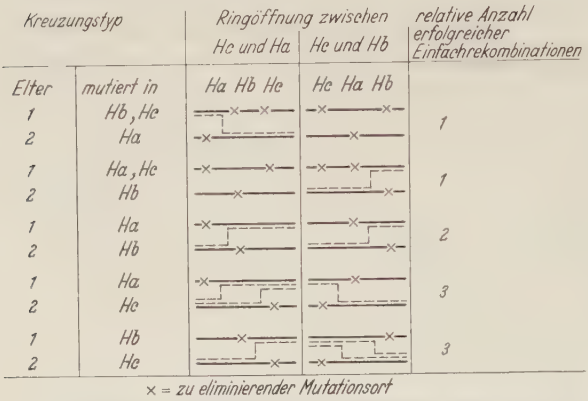


Abb. 7. Schema der erfolgreichen Einfachrekombinationen in verschiedenen Kreuzungen bei alternativer Öffnung des Ringes

b) Kreuzungen mit *Ha* × *Hb* geringere Werte als Kreuzungen mit *Ha* × *Hc* bzw. *Hb* × *Hc* hervorbringen und

c) Kreuzungen mit Doppelmutanten (mutiert in *Hb*, *Hc* und *Ha*, *Hc*) gleiche und um etwa die Hälfte niedrigere Werte als Kreuzungen *Ha* × *Hb* zeigen müssen.

Die gemittelten Werte dieser Rekombinantenhäufigkeiten betragen 470, 290, 100 (s. Tabelle 8 und 10).

Die Befunde könnten also sowohl mit der Existenz hoher negativer Interferenz, als auch durch eine Ringstruktur des Genoms mit einem Öffnungsvorgang an zufälligen oder gleichhäufigen, aber spezifischen Stellen in Einklang gebracht werden.

Summary

A selective system for host-range recombinants, similar to the negative host-range system in T 2, STREISINGER 1956, could be developed in ΦX 174:

In successive steps with wildtype phage plated on *E. coli C* a one-, two- and threestep bacterial mutant to resistance and their corresponding host-range mutants were isolated. By treating the triple host-range mutant with nitrite revertants could be obtained which had lost the ability of forming plaques on the triple mutant host bacteria.

Crosses with these revertants resulted in recombinants in many cases, able to grow on the triple mutant host bacteria. By spot tests these revertants could be grouped into three classes. Revertants which did not recombine in the spot test were attached to the same class. Only revertants belonging to different classes yielded recombinants in spot tests. Ordinary crosses showed remarkable differences in the frequency of recombinants. Frequencies of recombinants in inter-class-crosses were between $1—5 \cdot 10^{-4}$, whereas intra-class-crosses showed recombinants too, with a frequency of $1—7 \cdot 10^{-5}$.

Recombination data of inter- and intra-class-crosses made it probable that the distances between the mutations are nearly additive. Crosses with double-mutants led to the conclusion that there exists high negative interference (double and single recombinations are nearly at the same frequency) or that the genome has a circular structure which can be opened for replication at several points. The latter point is discussed in detail.

Für die Stellung der Aufgabe, zahlreiche Diskussionen sowie kritische Durchsicht des vorliegenden Manuskriptes bin ich Professor C. Bresch zu größtem Dank verpflichtet. Außerdem danke ich Dr. H. Bremer für Ratschläge und freundliches Interesse.

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THE PATTERN OF MUTAGEN-INDUCED BACK MUTATIONS IN *SALMONELLA TYPHIMURIUM**

By

Paul Margolin and Frank H. Mukai**

(Received July 24, 1961)

The elucidation of the purine-pyrimidine pair sequence of the DNA comprising a "gene" is one of the steps required for understanding how genetic information is carried. Pontecorvo (1958) has described the possibility of using specifically acting mutagens to determine base pair structures at genetically mapped sites of mutations. Freese (1959a, 1959b) has performed extensive studies along these lines with bacteriophage. He has inferred the nature of the mutational changes at many sites from the manner of response to induction of reversions by various mutagenic agents.

In the studies described here, leucine auxotrophs of the bacterium, *Salmonella typhimurium*, were treated with several mutagenic agents. These mutants had been mapped by complementation (abortive transduction) and 3-point recombination (complete transduction), and were found to fall into four complementation groups (Margolin 1959).

Materials and Methods

All 43 leucine mutants[1] used in this work were derived from *S. typhimurium* strain LT2. Of these, 28 were spontaneous and 15 were 2-aminopurine-induced. The mutagenic agents employed were ultraviolet irradiation (UV), diethyl sulfate (DES), and 2-aminopurine (AP). The minimal medium (M) used was that of Davis and Mingioli (1950), with the addition of 1.5% agar for the preparation of solid medium. Suboptimally enriched minimal medium (SEM) had 1.25% nutrient broth added.

For the experiments with UV, an overnight, aerated culture of each mutant tested was grown in nutrient broth. This was centrifuged, resuspended in saline, and irradiated to permit about 10% survival. A total of about 2×10^8 survivors were plated on SEM agar.

The experiments with DES and the initial test with AP involved spot tests similar to those described by Iyer and Szybalski (1958). From overnight, aerated broth cultures 0.1 ml. was plated and one drop of the mutagen placed on a paper disc at the center of the agar surface. Both M and SEM agar plates were used. All the mutant strains were tested in duplicate at least twice.

For the experiments involving AP treatment of the mutants in liquid medium, logarithmic growth phase cultures at a cell concentration of approximately 10^8 bacteria/ml were suspended in M medium containing 40 γ leucine/ml and 300 γ AP/ml. For those strains which gave a negative response to AP by the spot test method, the concentration of the mutagen was increased to 500 γ/ml. The cultures were incubated with aeration at 37° C for 75 minutes, washed once by centrifugation, resuspended in $^1/_5$ volume of M medium, and plated in triplicate by spreading 0.2 ml on M agar and on minimal agar suboptimally supplemented with 1 γ leucine/ml (SLM agar). The plates were then incubated at 37° C for 36 hours before scoring for reversions. Scoring was made at this time, since suppressor mutations usually appear later (Smith-Keary 1960).

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** Postdoctoral Research Fellow, Public Health Service, National Cancer Institute.

[1] Dr. M. Demerec kindly supplied many of the mutants.

Presumed reversions were distinguished from suppressor mutations by colony size. This method proved reasonably accurate when samples were subjected to preliminary genetic analysis. All those selected as reversions proved to be linked by transduction to the arabinose region of the *Salmonella* "chromosome" as would be expected of true reversions. Caution is necessary in interpreting these results, however, since a more extensive analysis is necessary to rule out the possibility of closely linked suppressor mutations such as those described by Dawson and Smith-Keary (1960).

Results and Discussion

The results of the treatments with UV and spot tests with DES and AP are presented in Table 1. It should be noted that while the spot test method appeared

Table 1. *The inducibility of reversions of leucine mutants by UV, diethyl sulfate and 2-aminopurine. The number of plus signs in the body of the table is an estimate of the degree of response. These estimates are relative to the maximum response for each particular mutagen*

Complementation group	Spontaneous mutants					AP induced mutants				
	Mutant strain No.	Spontaneous reversion frequency/10^{10}	UV	DES	AP	Mutant strain No.	Spontaneous reversion frequency/10^{10}	UV	DES	AP
I	10	2.0	−	++	(±)	147	5.0	×	+	(±)
	27	0.1	−	+	±	149	7.5	×	+	+
	32	1.5	−	+	(±)	150	60.0	×	++	(±)
	33	1.0	×	+	(±)	153	3.0	×	+	(±)
	46	89.0	×	+	+	158	10.0	×	++	(±)
	49	6.0	+	+	±					
	120	12.0	+	++	++					
	121	0.5	−	−	−					
	125	5.0	±	+	±					
	132	2.0	×	−	−					
II	129	2.0	+++	−	−	140	21.0	++	+++	(±)
						148	13.0	×	++	+
III	20	3.0	++	+++	+++	143	10.0	×	++	(±)
	25	2.5	×	++	+	146	2.5	×	+	+
	26	8.0	×	+++	+++	151	25.0	×	+	+
	34	4.0	×	+	±	155	17.0	×	++	(±)
	36	5.0	−	++	+	156	12.0	×	++	++
	44	8.0	×	+++	+++	157	50.0	×	+	+
	48	15.0	×	+++	+++	159	20.0	×	++	±
	122	11.0	+	+++	++					
	123	15.0	±	++	+					
	126	1.0	−	−	−					
	127	3.5	++	+++	+++					
	130	3.0	++	+++	+++					
	131	22.0	−	+	−					
	133	5.0	×	+	−					
	134	3.0	×	+++	++					
IV	21	12.0	+++	+++	+	152	20.0	×	++	+
	128	15.0	−	±	−					

× Not tested, UV Ultraviolet irradiation, DES Diethyl sulfate, AP 2-Aminopurine.

to be quite sensitive in detecting reversion responses to DES, it proved to be rather insensitive for AP. All those with negative responses to AP had to be further tested by treatment in liquid medium to determine if they were truly

resistant to back mutation induction. In those cases where negative spot tests were contradicted by results from AP treatment in liquid, a plus-minus in parenthesis, (±), is shown in Table 1.

Of the 28 spontaneous mutants tested with these few mutagens only three were resistant to reversion induction. Of these, only two were tested with all three agents. All 15 AP-induced mutants could be induced to revert by the agents used. It seems likely that further tests of these leucine auxotrophs with additional mutagens will leave few, if any, „mutagen stable" mutants such as have been previously described in both *Escherichia coli* and *S. typhimurium* (DEMEREC 1954, HARTMAN 1956). The results in Table 1 indicate that the frequency of spontaneous reversion is not related to the degree of inducibility by these mutagens.

It can also be seen that all mutants which show a response to AP also respond to DES, although the reverse is not true. All mutants which originated by AP induction proved to be sensitive to reversion induction by both DES and AP. There does not seem to be any obvious relationship between the way in which the mutants respond to UV treatment and the type of responses to DES and AP. The number tested with all three agents, however, is rather small, so that it is premature to draw any definite conclusions from this apparent lack of correlation.

The mutagenic effect of certain base analogues has been attributed to base pairing mistakes as a result of direct incorporation of the analogues into DNA (FREESE 1959, KIRCHNER 1960, RUDNER 1960, 1961). Since AP, in its common tautomeric form, is presumed capable of pairing with either thymine (T) or cytosine (C), this mutagen should produce transitional changes of adenine-thymine (A—T) nucleotide pairs to guanine-cytosine (G—C) pairs and vice-versa. Such transitions would result from AP incorporation by its pairing with one of the pyrimidine bases and then, in subsequent DNA replication, pairing with the alternate pyrimidine. This change would then be fixed during still later replications as a result of the pairing of the new pyrimidine with its usual purine partner. An example of this could be represented thus: G—C→AP—C→AP—T→A—T.

In studies with 5-bromouracil (BU) the evidence suggests that when BU replaces thymine, the A—BU pairs formed are functionally equivalent to A—T pairs (ZAMENHOF, DE GIOVANNI and RICH 1956). One may then consider whether the AP—C and AP—T pairs are functionally equivalent to either of the normal base pairs. Thus, when inducing reversions of auxotrophs with AP, the nucleotide pair containing this analogue may be functionally equivalent to the prototrophic configuration and may provide a revertant phenotype immediately. Further replications would only serve to complete the transition with normal bases. On the other hand, the initial base pair containing AP may be functionally auxotrophic, whereupon subsequent replications are needed to complete the transition which will result in the prototrophic phenotype.

From such considerations, there arises the possibility of obtaining two classes of mutation induction by AP. These would be distinguished by the presence or absence of a revertant phenotype immediately following treatment. The two types could be recognized by controlling the division of the cells following a short exposure to the mutagen. For those auxotrophs which have the prototrophic

phenotype as soon as AP is incorporated, the frequency of induced reversions should show no dependence on the number of divisions permitted by enrichment of the medium. For the second class, since the attaining of prototrophy must be preceded by divisions, enrichment of the medium should increase the reversion frequency.

The following experiments[1] were performed to determine if two such classes of response existed among the group of mutants showing such varied combinations of responses to the three mutagens (Table 1). The 43 leucine auxotrophs were treated with AP in liquid medium and plated on M and SLM agar. Demerec and Cahn (1953) have demonstrated that the number of divisions bacteria will undergo on a given medium can be regulated by varying the number of cells plated. In our experiments, auxotrophs underwent approximately two divisions on M agar and at least five divisions on SLM agar. To avoid possible variations due to differences in total amount of enrichment per plate, measured quantities (29 ml/Petri dish) of the two types of agar media were used.

The results presented in Table 2 demonstrate that the two postulated classes of response do indeed exist. Among the 21 mutants of spontaneous origin which respond to AP, 12 showed no increase in reversions on SLM agar (division independent class) over that on M agar. On the other hand, 9 of the mutants exhibited an increase in frequency of reversions on SLM agar (division dependent class). This demonstration of the dependence on division for the expression of potential back mutations differed from the "Witkin effect" (Witkin 1956, Doudney and Haas 1959). In our case, there is no requirement for a pool of amino acids (the concentration of a single amino acid, leucine, serves to control divisions) and there is no rapid decay of potential mutations with time (unpublished data).

Interestingly, all but 2 of the 15 mutants of AP induced origin belong to the division dependent class. This fact suggests that AP preferentially induces transitions of a particular type as a consequence of preferential pairing with one of the two pyrimidine bases.

Presumably those spontaneous mutants which also exhibit division dependence result from the same type of transition. Since, by definition, transitions lead to establishment of the alternative base pairs, these mutant forms must now contain the less favored pyrimidine. Such auxotrophs would be expected to provide a relatively poor response to reversion by AP. Conversely (by the same arguments) the division independent auxotrophs must now contain the preferred pyrimidine and should be reverted by AP with a high frequency. These predictions appear to be borne out by the data in Table 2.

Finally we wish to call attention to the comparatively high percentage of spontaneous mutants of complementation group III which are highly inducible by DES and AP and belong to the division independent class. Even among the AP induced mutants, the only two in this class are members of group III. Currently, when the specificity of mutagenesis is generally accepted as a characteristic of discrete sites it may be anachronistic to consider this character from the point of view of a complementation group or cistron. In fact, this situation

[1] Certain aspects of this portion of the work were initiated by one of us (FHM) during the tenure of a Postdoctoral Fellowship from the National Cancer Institute, under the sponsorship of Dr. George Streisinger.

Table 2. *The effect of increased divisions on the frequency of reversions induced by 2-aminopurine*

Complementation group	Spontaneous mutants						AP induced mutants					
	Mutant strain No.	Reversions/10^8 cells plated					Mutant strain No.	Reversions/10^8 cells plated				
		Control		AP				Control		AP		
		M agar	SLM agar	M agar	SLM agar	Division response		M agar	SLM agar	M agar	SLM agar	Division response
I	10	0.0	0.4	2.9	8.8	dep.	147	0.8	1.0	4.7	18.1	dep.
	27	0.0	0.0	18.7	24.6	ind.	149	0.8	0.5	29.5	65.0	dep.
	32	0.0	0.0	3.1	10.9	dep.	150	1.6	2.7	7.3	46.1	dep.
	33	0.0	0.2	2.0	2.2	ind.	153	0.1	0.2	1.6	15.9	dep.
	46	0.0	0.4	2.0	27.0	dep.	158	0.5	0.0	3.1	10.2	dep.
	49	3.2	3.6	6.5	21.1	dep.						
	120	0.0	0.8	97.7	100.1	ind.						
	121	0.0	0.3	0.0	0.0	–						
	125	0.0	0.4	5.8	22.9	dep.						
	132	0.0	0.3	0.0	0.4	—						
II	129	0.0	0.0	0.0	0.4	—	140	0.2	0.7	5.5	19.8	dep.
							148	0.2	0.2	10.0	38.0	dep.
III	20	0.0	1.9	233.5	226.2	ind.	143	0.4	2.4	13.3	46.1	dep.
	25	0.2	0.0	1.0	3.6	dep.	146	0.4	0.2	12.7	26.0	dep.
	26	3.8	3.5	286.7	293.9	ind.	151	0.5	0.9	45.7	53.8	ind.
	34	0.5	0.9	17.0	29.0	dep.	155	0.0	0.7	6.3	16.2	dep.
	36	3.7	2.3	36.6	50.0	dep.	156	0.8	1.5	127.5	119.8	ind.
	44	0.3	1.4	370.9	379.1	ind.	157	1.0	1.0	16.4	28.9	dep.
	48	2.2	2.5	181.3	196.6	ind.	159	1.8	2.6	7.7	25.5	dep.
	122	0.6	1.8	230.6	230.2	ind.						
	123	0.0	1.2	22.2	24.6	ind.						
	126	0.0	0.3	0.0	0.3	—						
	127	2.0	2.3	256.0	254.2	ind.						
	130	0.0	0.8	187.1	194.1	ind.						
	131	9.0	10.8	7.3	9.3	—						
	133	0.3	0.3	0.5	1.2	—						
	134	39.1*	40.1*	362.1	387.0	ind.						
IV	21	0.8	0.6	9.7	29.0	dep.	152	0.7	0.1	5.5	37.6	dep.
	128	0.4	1.0	0.6	1.9	—						

dep. = Division dependent, ind. = Division independent.

* High figures here is attributed to the introduced spontaneous mutants which must have arisen in the overnight culture. (Compare with figures in Table 1.)

may merely reflect a somewhat unusual molecular makeup. The accumulation of additional auxotrophs should clarify this situation.

Preliminary results with other mutagens, not discussed in this paper, tempt us to specify which base pairs are actually involved in each of the two classes of response. However, we feel this would be premature on the basis of presently available experimental evidence.

Summary

The effects of UV, diethyl sulfate, and 2-aminopurine in causing back mutations were studied with spontaneous and 2-aminopurine-induced leucine auxotrophs of *Salmonella typhimurium*. A correlation was noted between the effectiveness of diethyl sulfate and 2-aminopurine. All the 2-aminopurine inducible mutants fell into two categories; those whose frequency of induced reversions were independent

of the number of cell divisions following treatment and those whose frequencies were strongly dependent upon division. It was suggested that the two categories represented the two purine-pyrimidine base pairs.

Almost all the 2-aminopurine induced mutants belonged to the division dependent category. From this it was inferred that 2-aminopurine paired preferentially with one of the two pyrimidine bases.

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MUTATION AS AN ERROR IN BASE PAIRING

I. THE MUTAGENICITY OF BASE ANALOGUES AND THEIR INCORPORATION INTO THE DNA OF *SALMONELLA TYPHIMURIUM* * **

By

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With 7 Figures in the Text

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A. Introduction

A gene may be conceived of as a unit of function composed of a linear sequence of many nucleotides. The mutational sites that are recombinable within the gene involve just one or a few nucleotide pairs (BENZER 1957, PONTECORVO 1958). The precise linear sequence of the nitrogenous bases is believed to determine the specificity of the gene. It follows that a mutation results from any permanent alteration in the sequence of nucleotide pairs. Experiments with nitrous acid have, in fact, shown that a single chemical alteration is sufficient to produce a mutagenic change (SCHUSTER and SCHRAMM 1958; MUNDRY and GIERER 1958; FREESE 1959; KAUDEWITZ 1959; LITMANN and EPHRUSSI-TAYLOR 1959). Thus, the unit studies in point mutations may be at least as small as a single nucleotide.

The use of mutagenic base analogues known to be incorporated into DNA and of agents which produce unusual bases in nucleic acid *in situ* can provide information on the molecular changes that result in mutations. When applied in a comparative manner to a well mapped gene, the mutagenic specificity of a given base analogue can be determined. In addition, an analysis of the chemical events that occur from the time of treatment to the expressed mutation should ultimately lead to the identification of the nucleotide paris constituting a mutational site. Experimental evidence will be presented following these theoretical considerations. The base analogues used in these studies were 5-bromouracil (BU) and 2-aminopurine (AP). These agents are believed to act as tautomeric mutagens and to produce transitional changes (FREESE 1959a, b, c), *i.e.*, replacement of an adenine-thymine nucleotide pair by a guanine-cytosine pair, or *vice versa*.

This paper deals with induced reversions from auxotrophy to prototrophy in *Salmonella typhimurium* in the presence of BU and its deoxy-derivative BUDR, AP and nitrous acid. Twenty-three mapped mutational sites in the tryptophan-*D* and -*C* loci, known to block the last step in the biosynthesis of that amino acid, were selected for these studies (RUDNER and BALBINDER 1960; BALBINDER 1961). Although the number of mutants analyzed is relatively small the results obtained

* I dedicate this and the following paper to my teacher, Prof. L. C. DUNN.

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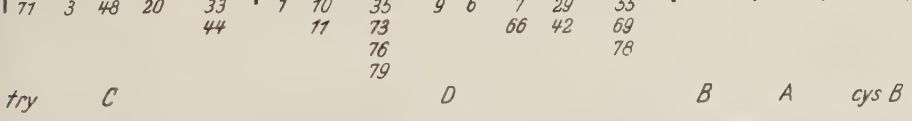
*** Public Health Service Training Fellow.

parallel those found by Freese (1959c), inasmuch as the tryptophan loci contain base analogue-inducible and non-inducible sites. Correlated with these genetic findings, chemical studies reveal that a large amount of BU can be incorporated into the DNA by replacing thymine specifically; this confirms earlier reports by Dunn and Smith (1954) and Zamenhof *et al.* (1954). On the other hand, only a small amount of unaltered AP was recovered from the DNA of a purine-requiring mutant. The amount of AP was too small to determine the actual per cent replacement of individual purine(s). It is interesting that although the per cent incorporation of AP is smaller than the incorporation of BU, the former is more effective as a mutagen than the latter. This supports the theory that permanent mutations are not caused merely by the replacement of a DNA base by its base analogue. Rather, mutations result from mistakes in base pairing in which the analogue may engage more or less frequently. It would therefore seem that the rare incorporated AP molecules have a greater chance of making erroneous pairings than the frequently incorporated BU molecules. The accompanying paper presents the results of the kinetic studies aimed at the elucidation of the mechanism of BU- and AP-induced mutagenesis.

B. Materials and Methods

I. Bacterial strains

Twenty-three tryptophan requiring mutants (*try*$^-$) of *Salmonella typhimurium* strain *LT-2* or a modified *LT-7* (see later) were obtained through the kindness of Drs. M. Demerec and E. Balbinder. These mutants are altered at the *tryC* and *tryD* loci which control the last step in the biosynthesis of that amino acid. The two adjacent loci are part of a four-locus sequence that controls the biochemical pathway of tryptophan synthesis. Demerec and Hartman (1959) found that the order of the four loci and a linked cysteine locus is as follows: *tryC—tryD—tryB—tryA—cysB*, which agrees with the sequence found in *E. coli* (Yanofsky 1960). Both *tryD* and *C* control the synthesis of the enzyme tryptophan synthetase. The *tryD* locus controls the formation of the protein B component which is primarily concerned with the reaction, indole→tryptophan, and the *tryC* locus controls component A which is involved primarily with the reaction, indoleglycerol phosphate→indole. The mutant strains are divided into four groups with respect to their origin: (1) spontaneous; (2) ultra-violet induced; (3) AP-induced, and (4) mutants that arose spontaneously in strain *LT-7* carrying the mutator gene (*mut*); some are still *LT-7*, containing a proline segment and the mut$^+$ gene from *LT-2* (Miyake 1960), and some are now *LT-2* by transduction of the *try*$^-$ marker away from the mutator strain (see Table 3). All twenty-three mutants were mapped by Dr. E. Balbinder (1961), who employed complete transduction tests either in two- or three-point cross with the linked genes *cysB* and *tryB-4* (the latter can grow on indole unlike the *C* and *D* mutants). The order of the mutants is as follows:



In the case that mutant numbers are aligned, the exact order was not determined although they do recombine with each other. Three sets of mutants, *tryD-11* and *10*, *tryD-7* and *66* and *tryD-29* and *42*, did not recombine with each other in reciprocal transduction tests; thus each pair seems to be an alteration at the same site (Balbinder 1960).

For the chemical incorporation of AP a variety of *LT-2* purineless (*pur-102*, *-106*, *-107*, *-108* and *-109*), adenineless (*ade-91*, *-97*) and one guanineless (*gua-12*) mutants were tested.

These strains were also obtained from the collections at the Carnegie Institute of Washington at Cold Spring Harbor. After screening them with respect to their growth pattern, *pur-107* was selected for the chemical investigations. The strain grows on both adenine and guanine; when starved for these purines it releases a brown substance which resembles the substance secreted by the purple mutants of *Neurospora*. The biochemical block seems to be in the conversion of 5-amino-4-imidazolecarboxamide ribotide (AICAR) to inosinic acid (IMP).

II. Media

Synthetic minimal medium (MM) — per 1 liter distilled water: 10.5 g K_2HPO_4; 4.5 g KH_2PO_4; 1 g $(NH_4)_2SO_4$; 0.47 g Na-citrate; 0.05 g $MgSO_4$ and 2 g glucose. For culturing the various *try*$^-$ mutants 20 μg L-tryptophan (Sigma)/ml was added. This minimal medium allowed the bacteria to reach a titer of *ca.* 2×10^9 cells/ml when grown at 37°C with aeration.

Plates contained either nutrient-agar plus 0.5% agar (Difco) supplemented with tryptophan (these were used for viable counts) or synthetic medium plus 2% agar and 0.5% glucose. The minimal plates used for reversion studies were usually divided into three groups: (1) Unsupplemented (MM); (2) Supplemented with 0.03 μg tryptophan/ml (Try$^{\pm}$MM) and (3) enriched with 0.01% nutrient broth (enMM). Both kinds of supplements allowed *ca.* 5×10^8 *try*$^-$ bacteria to replicate once on the plates.

Special media. 1. A modified F_8 *medium* for BUDR mutagenesis (FREESE 1959b). Synthetic medium which contains per ml the following metabolites: 20 μg L-glycine; 40 μg DL-methionine; 10 μg L-leucine; 10 μg L-valine; 20 μg DL-serine; 10 μg adenine; 10 μg guanine; 5 μg uracil; 10 μg deoxycytidine; 0.2 μg calcium pantothenate; 0.2 μg thiamine and 0.2 μg pyridoxine. To this 200 μg BUDR/ml and 200 μg aminopterin/ml were added. Or the same concentration of BUDR was used in the presence of 10 μg 5-fluorodeoxyuridine/ml (FUDR) with 0.2% vitamin-free casamino acids instead of the above listed metabolites.

2. *H-medium* for the chemical incorporation of AP. Synthetic medium supplemented with 0.4% glucose; 40 μg adenine; 10 μg L-histidine; 0.25 μg thiamine and 500 μg AP/ml.

3. *Enriched medium* for the chemical incorporation of BU, as described by ZAMENHOF *et al.* (1956a).

III. Analogues and other chemicals

Aminopterin (California Found.) after autoclaving was kept dark in the cold as a 2 mg/ml solution in $^1/_{100}$N-NaOH.

5-fluorodeoxyuridine (FUDR), obtained as a gift from Hoffman-La Roche Inc., was kept in the cold as an aqueous solution of 1 mg/ml.

5-bromouracil (BU) and *5-bromodeoxyuridine* (BUDR) (California Found.) were kept in the cold as aqueous solutions of 2 mg/ml.

2-aminopurine (AP) (Sigma Co.) since the compound is heat labile it was not autoclaved but was either sterile-filtered as an aqueous solution of 2 mg/ml or the weighed cyrstals were dissolved directly in sterilized warm synthetic media (not higher than 40°C) at a concentration of 300 μg/ml.

Nitrous acid-$NaNO_2$ (M.W. = 69.01) was dissolved in either 1M acetate buffer at pH 4.6–4.7 or in 0.2M acetate buffer at pH 4.2 to yield 1N or 2N $NaNO_2$ solutions. The buffer solutions were autoclaved in small flasks; while still warm the sodium nitrite crystals were added and the flasks were immediately sealed with cotton plugs and foil to prevent the escape of NO_2 gas.

IV. Biological assays

Assays for the mutagenicity of nitrous acid were conducted by the paper-disc method of IYER and SZYBALSKI (1958). Filter-paper disks (Whatman No. 1), 1.5 cm in diameter and saturated with the mutagenic agent ($NaNO_2$-acetate buffer) and the various controls, were placed on the surface of minimal agar (MM, Try$^{\pm}$MM and enMM) plates which were seeded with *ca.* 5×10^8 washed *try*$^-$ cells. The plates were incubated for 48 hours at 37°C. Assays for the mutagenicity of AP and BUDR were carried out in liquid cultures. Each mutant strain was grown in the presence of the mutagen for about 10 divisions at 37°C with aeration in minimal medium with the appropriate supplements. Following growth the cell suspension was washed twice in saline (0.9% NaCl) and plated for viable and mutant counts on the various agar media.

V. Chemical procedures

1. Isolation of highly polymerized DNA by a modified method described by Zamenhof *et al.* (1956a). The cells were harvested by 1 hour centrifugation at 1900 × G and washed six times with 0.9% NaCl. The packed cells (8–12 g wet weight) after overnight storage in the deep freeze were resuspended in 10 ml of distilled water by means of a glass tissue homogenizer and the suspension was poured into a beaker containing 100 ml of 15% aqueous solution of Duponol (Delta Chemical) which was adjusted to pH 6.4. After incubation with periodic agitation at room temperature for 1 hour and in the cold for 4–5 hours the cells were completely lysed. To this lysate 2 volumes of 95% ethanol were added, and the crude fibrous precipitate was removed, washed in 75% ethanol, drained and redissolved in a tissue homogenizer with 20–30 ml 10% NaCl which contained $^1/_5$ volume of 0.1 M sodium citrate. After overnight storage in the cold, the white viscous solution was centrifuged in the cold for $^1/_2$ hour at 31,000 × G (Spinco Model L) and the supernatant was precipitated whith alcohol, washed and redissolved as described above (this process was repeated at least twice, during that time the previously collected supernatant was stored in the cold). For measuring the incorporation of BU into DNA the further removal of ribonucleic acid (RNA) is not required since BU replaces thymine specifically. However, for the incorporation of AP into DNA, RNA was removed by alkaline digestion either with or without dialysis using 0.3 N NaOH at 37°C for 16–20 hours. After removal of RNA, the extract was neutralized with 1 N HCl, the salt concentration adjusted to 10% NaCl and the DNA reprecipitated in 95% ethanol. The final DNA precipitate was drained and dried in a vacuum desiccator over P_2O_5. The yield was 3–4 mg of DNA per gram of wet weight of bacteria as determined by the Dische diphenylamine reagent.

Preparations of DNA (not highly polymerized) were also made by the method of Smith and Wyatt (1951). This procedure involves incubating washed bacteria for 15–20 hours at 37°C in a small volume of 1 N NaOH. Insoluble material was removed by centrifugation, the supernatant was brought to pH 4.0 with acetic acid and one volume 95% ethanol was added. The precipitate, containing DNA (free of RNA), was collected by centrifugation dissolved in dilute NaOH and the protein removed by surface gel formation on shaking with a mixture of 8 parts of chloroform and 1 part of commercial octanol (v/v). A further precipitation with acetic acid and ethanol completed the preparation of DNA.

2. Hydrolysis of DNA. *a) Hydrolysis to purines and pyrimidines.* 10 mg dry DNA was hydrolyzed for 1 hour in 0.05–0.1 ml 7.5 N perchloric acid (PCA) at 100°C (Marshak and Vogel 1950). The same volume of water was then added. The mixture was centrifuged and the brown clear supernatant fluid then contained free bases.

b) Hydrolysis to purines only. To a solution of 10 mg DNA 1 N HCl was added to yield a 0.1 N HCl solution and the mixture incubated at 37°C for 18–20 hours. After the pH was adjusted to neutrality with 1 N NaOH the mixture was centrifuged. The supernatant consisted of free purines (Chargaff 1955).

c) Enzymatic hydrolysis to 5′-deoxynucleotides. Deoxyribonuclease (Worthington Biochemical Co.) (10 μg/ml) was added to a solution of DNA (5 mg/ml) in ethanolamine-HCl buffer pH 9.4 containing 0.05 M $MgSO_4$. The solution was allowed to remain at room temperature for 30 minutes, during which time the DNA dissolved, its viscosity was reduced and presumably partial digestion occurred. Snake venom phosphodiesterase (0.3 optical density units at 280 mμ/5 mg DNA) was then added and the pH was adjusted to 9.4 with 0.02 M ethanolamine-HCl buffer. With the addition of 5 drops of chloroform the tube was sealed with a stopper and the reaction mixture incubated at 37°C for 24 hours (Price 1960). The purification of phosphodiesterase from Russell's viper venom (Ross Allen Reptile Institute) on a cellulose column was based on the studies of Hurst and Butler (1951) who found that phosphodiesterase and 5′-nucleotidase may adsorb from aqueous solutions onto packed cellulose fibers and that dilute solutions of neutral salts elute both of these enzymes. Fifteen mg of crytalline snake venom were dissolved in 5 ml water and poured into a cellulose column ($^1/_2 \times 6''$) which had been previously packed with water washed Whatman cellulose powder (Standard Grade). Eluents of water and 0.1% NaCl were then applied. The rate of flow through the column was 0.5—1.0 ml/min; successive 5 ml fractions were collected. The optical densities of the fractions were read at 280 mμ. The second peak in 0.1% NaCl

consisted of pure phosphodiesterase (the first peak in H_2O consisted of nucleotidase and was discarded).

3. Separation of nucleic acid components. The halogenated uracil (BU) was separated from the natural purines and pyrimidines in the perchloric acid hydrolysate of DNA by paper chromatography with the use of the following solvent systems:

1. n-butanol saturated with water (MARKHAM and SMITH 1949).

2. 6 vol. n-butanol plus 1 vol. 0.6N aqueous ammonia (CHARGAFF *et al.* 1951).

For the separation of mononucleotides from an enzymatic hydrolysate of DNA containing AP, the following solvent systems were used:

1. 10 vol. isobutyric acid plus 6 vol. 0.5N NH_4OH (MAGASANIK *et al.* 1950) all of the deoxynucleotides were completely resolved in this solvent.

2. Isopropanol 170 ml, concentrated HCl (sp. gr. 1.19) 41 ml and water to make 250 ml (WYATT 1951). In this solvent thymidylic acid and deoxycytidylic separate nicely from the purines which have become free bases due to the breakage of the N-glycosidic bond in acid.

3. n-butanol saturated with water, with ammonium bicarbonate added to the solvent in a small beaker and placed at the bottom of the tank. In this solvent deoxynucleotides do not travel at all. Therefore the solvent was used as a control to determine whether the enzymatic hydrolysis was successful.

For the routine analysis of DNA that contained base analogues a unidimensional descending chromatographic method was chosen. Fifteen to twenty aliquots of 5 λ were applied at the origin at a distance of 0.5 cm apart. The origin was drawn 8 cm from the edge of Whatman No. 1 or No. 50 filter paper (16 × 46 cm). The free base or mononucleotide bands were located on the paper by using an ultraviolet lamp with an appropriate filter. For clearer visualization of 260 mμ absorbing compounds U.V. fluoroscope sheets were used so as to mark the bands accurately (TSUBOI and PRICE 1959). The spots were cut out and eluted in 0.1N NCl for 16 hours at room temperature and for 3–4 hours at 37°C. The absorption spectrum (over a range of 230—340 mμ) of each eluate was read in a Beckman model DU spectrophotometer against the corresponding eluates of blanks. The readings were converted to molar concentrations using the absorption maxima extinction values or the differential extinction (Δ) values (VISCHER and CHARGAFF 1948). The ratios of extinction values at selected wave lengths (250/260, 280/260) were calculated for assessment of purity and identity of the isolated compounds.

C. Experimental

I. Induction of reversion in tryptophan requiring mutants

1. The mutagenic action of BUDR. Mutagenesis with BUDR in *Salmonella typhimurium* is achieved only when the bacteria are rendered thymine deficient or when a thymine requiring mutant is used. The analogue by itself without this requirement is ineffective except in slowing the general metabolism of the bacteria. To create the condition of thymine deficiency the folic acid analogue, aminopterin, was used (ZAMENHOF *et al.* 1958). Other investigators have resorted to the use of sulfanilamide, an inhibitor of p-aminobenzoic acid which is a constituent of folic acid (DUNN and SMITH 1957; LITMAN and PARDEE 1956, 1960). Both compounds are known to inhibit the methyl transfer in the synthesis of purines, pyrimidines and various amino acids. Specifically an interference with the endogenous methylation of deoxyuridylic acid is achieved (FRIEDKIN and KORNBERG 1957). Another useful agent for this purpose is 5-fluorodeoxyuridine (FUDR), an analogue known to inhibit the enzyme thymidylate synthetase (HEIDELBERGER *et al.* 1957; COHEN *et al.* 1958). The inhibition of bacterial growth by FUDR is reversed completely with thymidine and partially with BUDR (LORKIEWICZ and SZYBALSKI 1960). In order to reduce the general inhibition of aminopterin, a mixture of essential metabolites which contain the one carbon-containing fragments was added. Treatment of bacteria with aminopterin or

FUDR alone had no mutagenic effect. Only the mixture of BUDR and aminopterin (250 μg/ml of each) or BUDR and FUDR (250 μg/ml and 10 μg/ml, respectively) was effective in producing reversions from auxotrophy to prototrophy. In earlier studies the free base BU was employed (Rudner and Balbinder 1960), but it was later found that BUDR was a superior mutagen.

Under this condition (BUDR and aminopterin) the bacteria grow very slowly with a generation time of 120–150 minutes as compared to the normal generation time of 55–60 minutes. *Salmonella typhimurium*, unlike *E. coli*, appears to be more resistant to aminopterin. The bacteria do not die rapidly due to "thymineless death", nor do they develop into long threads as was observed for *E. coli*. (Cohen and Barner 1956; Zamenhof *et al.* 1956b; Okada *et al.* 1960). Each mutant strain was grown in minimal medium supplemented with BUDR, aminopterin and metabolites for approximately 10 divisions. Following this treatment the cells were washed twice in saline and $4–5 \times 10^8$ cells were seeded on the three kinds of minimal plates (MM, Try$^{\pm}$MM, enMM). Appropriate dilutions were also plated on nutrient agar plates for the total viable count. After 48, 72 and 96 hours the number of revertant colonies was scored. Only phenotypically wild type colonies, as determined by colony size on enriched minimal agar, were considered; partial reversion to prototrophy due to suppressor mutations at different loci gave rise to microcolonies which appeared late on the plates and were not analyzed (Balbinder 1960). The possibility still exists that these reverse mutations to the original phenotype did not arise by a back mutation at the mutant site, but rather by some other ("suppressor") mutation at a different or at the same locus.

The increased number of prototrophs was due to BUDR-induced mutations and not to the selection of spontaneous revertants present during growth in the liquid medium. This was established from three findings: (1) more revertants were always recovered when the treated bacteria were plated on the supplemented minimal agar (Try$^{\pm}$MM and enMM) as compared to when they were plated on unsupplemented agar (MM) (see Table 1); (2) revertants could be obtained by plating untreated cells directly on enriched minimal agar containing BU and aminopterin, and (3) the results of reconstruction experiments. The latter consisted of mixing a large population of tryptophan-requiring streptomycin-sensitive cells ($try^{-}s^{s}$) with a small number of tryptophan-independent streptomycin-resistant cells ($try^{+}s^{r}$) in the experimental condition described above. Every hour an aliquot was removed and the number of parental ($try^{-}s^{s}$) and mutant types ($try^{+}s^{s}$, $try^{+}s^{r}$) were determined by plating on appropriate agar plates (some of which contained 200 μg streptomycin/ml). If under these conditions the pre-existing revertants have a selective advantage and can propagate at a faster rate than the parentals, the increase of the introduced $try^{+}s^{r}$ bacteria should be faster than that of the $try^{-}s^{s}$ cells. If there is no selctive advantage then the $try^{+}s^{r}$ bacteria should increase at a constant rate which cannot be distinguished from that of $try^{-}s^{s}$. Indeed, the latter result was observed (Fig. 1). The introduced mutants increased at the same rate as the parentals, while the newly induced mutants $try^{+}s^{s}$ appeared only on the supplemented minimal plates (Rudner 1961). It was therefore concluded that the appearance of mutants during growth in the presence of the analogue could be attributed to the mutagenic action of BUDR and not to selective enrichment of mutants already present in

the culture. LITMAN and PARDEE (1956), who first observed the mutagenic action of BU in bacteriophage *T2*, also excluded the possibility of selection on the basis of one-step growth and single burst experiments.

2. The mutagenic action of 2-aminopurine. The mutagenic effect of AP does not require the interference with the biosynthesis of DNA precursors as was found for BUDR. It is effective upon simple exposure to growing bacteria. With increasing concentrations of AP one observes a linear increase in the number of *try*$^+$ prototrophs (Fig. 2). In the presence of

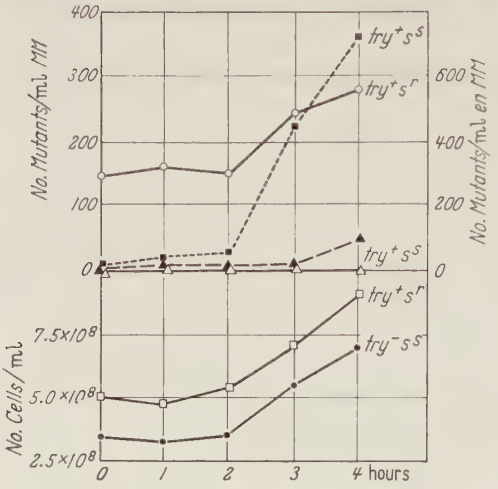


Fig. 1

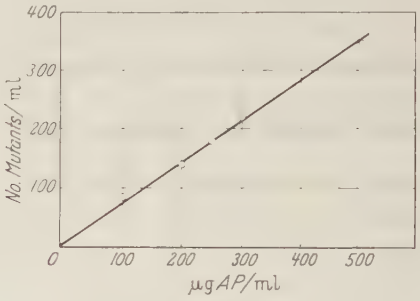


Fig. 2

Fig. 1. A reconstruction experiment performed in the presence of BUDR, aminopterin (200 μg/ml of each) and metabolites. The mutant strain *tryD-79* was employed; it is streptomycin sensitive (s^s). A resistant (s^r) strain (200 μg streptomycin/ml) was previously isolated. The increase of cell numbers in an artificial mixture of *try*$^+s^r$ (*ca.* 150 cells/ml) and of *try*$^-s^s$ (*ca.* 3×10^8 cells/ml) was determined; the increases were compared with each other and with the increase of *try*$^-s^r$ (□) when cultured alone. The introduced mutants (*try*$^+s^r$ = ○) increased at the same rate as the parentals (*try*$^-s^s$ = ●) in the artificial mixture. The newly induced *try*$^+s^s$ were determined by plating on the three kinds of minimal plates: △ = MM; ▲ = Try±MM and ■ = enMM. BUDR did not induce *try*$^+s^r$ as determined by replica plating onto agar containing 200 μg streptomycin/ml

Fig. 2. A dose response curve of *try*$^+$ induced mutants as a function of AP concentration. The mutant strain used in this experiment was *tryD-10*. Bacteria synchronized by fractional filtration were suspended in 5 flasks containing minimal medium with various concentrations of AP. The cells were allowed to undergo two DNA replications. The number of prototrophs was scored only from the MM plates. At 500 μg AP/ml, only 4×10^{-1} of cells survived and the numbers of induced *try*$^-$ were corrected for this death (see Fig. 7 in RUDNER 1961)

500 μg AP/ml tryptophanless, and especially, purineless cells developed into long "snake-like" threads and many were unable to proliferate (RUDNER 1961, Fig. 7). The generation time was also observed to increase with added increments of AP. For routine analysis each mutant strain was grown in the presence of either 300 μg or 200 μg AP/ml for approximately 10 divisions. The increased frequency of back mutations was of the order of 10^3–10^4. This represented a tenfold increase over that observed with BUDR.

It was further established that the appearance of prototrophs was solely the result of AP mutagenesis and not the selection of pre-existing wild types. The methods used to confirm this were identical with those used with BUDR. Fig. 3 illustrates a reconstruction experiment performed in the presence of AP. The generation times of the four cell types: *try*$^-s^s$, *try*$^-s^r$, *try*$^-s^s$ and *try*$^+s^r$, were indistinguishable when they were grown separately or in artificial mixtures in

the presence and absence of AP. We can therefore assume that the newly induced *try*$^+$ mutants do not proliferate faster than the parental cells. FREESE (1959b) also excluded selection of pre-existing rII mutants present in his bacteriophage T4 stock.

3. The mutagenic action of nitrous acid. Nitrous acid is an effective inactivating and mutagenic agent for RNA and DNA in *in vivo* and *in vitro* experiments (SCHUSTER and SCHRAMM 1958; MUNDRY and GIERER 1958; FREESE 1959c; TESSMAN 1959; KAUDEWITZ 1959, and LITMAN and EPHRUSSI-TAYLOR 1959). It rapidly deaminates the purine and pyrimidine residues of nucleic acid. Guanine, adenine and cytosine are converted to xanthine, hypoxanthine, and uracil respectively. The rates of mutant production, inactivation and deamination are all pH dependent. VIELMETTER and SCHUSTER (1960) found that the log of the mutation rate for r mutants of the coliphage *T2* decreases linearly with an increasing pH. In liquid medium bacteria exposed to sodium nitrite at a pH of 4.2 die within the first minutes of treatment. Assays for the mutagenicity of nitrous acid were therefore conducted by the filter-paper disk method (IYER and SZYBALSKI 1958). The concentration gradient (created by diffusion) resulted in the death of only those cells that were seeded near the paper disk. Two mixtures of different pH were tested: (1) 1N $NaNO_2$–0.2 M acetate buffer (pH 4.2) and (2) 1N $NaNO_2$—1M acetate buffer (pH 4.6), solutions of 1N $NaNO_2$—H_2O and acetate buffer served individually as controls. It has been reported that under conditions of low pH or high temperature (pH 4–5 or 45°C) mutagenesis occurs probably through depurination of DNA (ZAMENHOF and GREER 1958; GREER and ZAMENHOF 1959; FREESE 1959c). A slight effect of low pH was observed in the control treatment (acetate buffer at pH 4.2 or 4.6) as a 3–5 fold increase in back mutation frequency. The number of revertant colonies recovered on the control plates was therefore subtracted from the number on the nitrous acid plates.

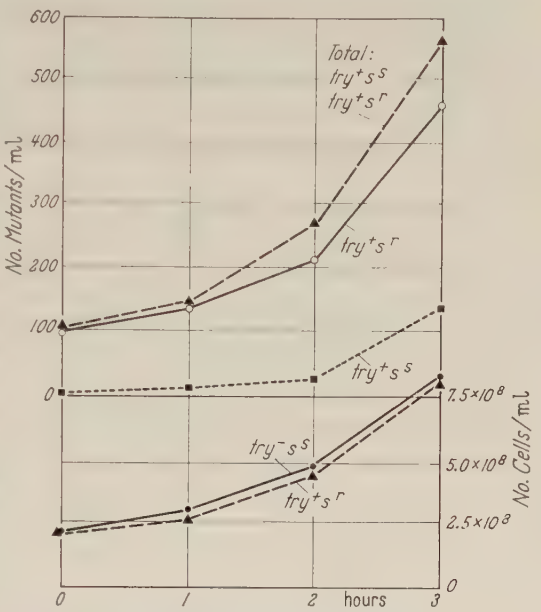


Fig. 3. A reconstruction experiment performed in the presence of 200 μg AP/ml employing mutant strain *tryD-10* which is streptomycin sensitive (s^s). A resistant (s^r) strain (200 μg streptomycin/ml) was previously isolated. The increase of cell numbers in an artificial mixture of *try*$^+s^r$ (*ca.* 100 cells/ml) and of *try*$^+s^s$ (*ca.* 2.5×10^8 cells/ml) was determined; the increases were compared with each other and with the increase of *try*$^+s^r$ when cultured alone (▲). The introduced mutants (*try*$^+s^s$ = ○) increased at the same rate as the parents (*try*$^-s^s$ = ●) in the artificial mixture. The total number of introduced and AP induced *try*$^+$ (▲) mutants was determined by plating on MM. Replica plating onto agar containing 200 μg streptomycin/ml were performed so as to distinguish between *try*$^+s^s$ = ■ and *try*$^+s^r$

The treatment consisted of growing each mutant strain to full turbidity (*ca.* 2×10^9 cells/ml) in minimal medium. Washed cells in 0.9% NaCl were allowed to stand at room temperature for a few hours. Killing was greatly reduced when metabolically inactive cells were treated (KAUDEWITZ 1959b). Supplemented minimal plates were seeded with $4-5 \times 10^8$ cells and allowed to dry for 30 minutes at 37°C. After this interval paper discs soaked in the three solutions were placed on the surface at the center of the enriched minimal plate and incubated at 37°C for 48 hours. Survival was checked by spreading the same number of cells on unsupplemented minimal plates. After 48 hours incubation the surviving cells were washed off the plates and the viable number was determined. The fraction surviving was 1.3×10^{-2}; 5.4×10^6 cells/plate remained viable. The zone of toxicity was found to be 1.2—1.5 cm from the periphery of the paper disc. In this zone all the cells were killed. Mutant colonies (*try*$^+$) appeared in a ring around source of the toxicity. No revertant colonies developed on the unsupplemented minimal plates. This indicated that several cell divisions (specifically DNA replications) are necessary after treatment in order to obtain phenotypically wild type colonies.

Table 1. *The number of wild type colonies (try*$^+$*) recovered on the various minimal plates (MM, Try*$^{\pm}$*MM and enMM) after treatment with AP, BUDR and nitrous acid*

The numbers represent average values calculated from 3 plates after 72 hours incubation at 37°C. All mutagenic treatments were performed in liquid except for nitrous acid which was done directly on the plates with paper disks.

Mutant strain	Treatment	No. colonies per plate		
		MM	Try±MM	enMM
C-48	AP	52.0	90.0	164.0
	BUDR	27.0	92.0	337.0
	HNO_2	1.0	44.0	152.0
	Control	1.5	2.5	7.5
D-73	AP	48.0	75.0	96.0
	BUDR	11.0	25.0	101.0
	HNO_2	0.0	22.0	72.0
	Control	1.5	5.0	7.5
C-3	AP	116.0	190.0	247.0
	BUDR	6.0	13.0	80.0
	HNO_2	2.0	17.0	544.0
	Control	0.7	1.5	4.0

The number of revertant colonies scored on the three kinds of minimal plates presents a problem for interpretation. Table 1 illustrates the results for three mutant strains after the various mutagenic treatments. As mentioned earlier, both enrichments, 0.01% nutrient broth and 0.03 μg tryptophan/ml, allow 5×10^8 cells to divide once. There is nevertheless a great discrepancy in the values obtained. The assays for the mutagenicity of 2-aminopurine yielded reasonable proportions among the MM, Try$^{\pm}$MM and enMM plates, while the assays for the mutagenicity of BUDR resulted in a tenfold or greater difference between the numbers on MM and enMM. The same was true for the nitrous acid experiments and in addition there was a great discrepancy between the two types of enriched plates. For the nitrous acid data a plausible explanation is that the amino acids and other metabolites present in the broth protect the cells from being inactivated. Their presence could reduce the amount of oxidative deamination *in situ* and therefore more cells, including newly induced mutants, would survive. The results with BUDR are analogous to WITKIN's (1956) findings for ultraviolet-induced reversions in *Salmonella* and *E. coli*. The maximal yield of U.V.-induced prototrophs was determined irreversibly by the amount of nutrient broth added to the agar or to the post irradiation culture medium. Furthermore, the required

growth factor alone was inactive as were mixtures of vitamins or purines and pyrimidines. However, a mixture of amino acids, including a limiting amount of the required growth factor, had the same effect as nutrient broth in promoting the maximal yield of induced prototrophs. Protein synthesis is considered necessary for the fixation of the mutation induced with ultraviolet light (Witkin 1956; Doudney and Hass 1959). On the other hand, immediate protein synthesis is not required for the fixation of the mutation induced with alkylating agents (Strauss and Okubo 1960), BU and AP (Litman and Pardee 1959; Brenner and Smith 1959; Nakada *et al.* 1960) and with caffeine (Glass and Novick 1959). It appears that some other metabolic process leads to an increase in the number of revertants induced by chemical mutagens. Although the number of prototrophs scored on MM and Try$^{\pm}$MM represents an actual increase in the mutation frequency, it is only a fraction of the real numbers induced by BUDR. Perhaps the broth enrichment is necessary to repair some cellular damage caused by BUDR in some of the potentially mutant cells. Hence, they remain as cryptic mutants on the MM and Try$^{\pm}$MM plates and can express themselves as phenotypically wild type colonies only on enriched minimal plates.

Table 2. *Inductions of reverse mutation with base analogues and nitrous acid in twenty-three alleles at two tryptophan loci (D and C) of Salmonella typhimurium*

In the table + signifies an increase of back mutation frequency of tenfold or more — means that there was less than tenfold increase. Fifteen out of twenty-three mutant strains, or 65%, were base analogue inducible.

No. of Strains	No.	HNO_2	AP	BUDR
3, 48, 44, 1, 35, 73, 76, 66, 70, 69	10	+	+	+
10, 79	2	—	+	+
7	1	+	—	+
33	1	+	+	—
78	1	+	—	—
71, 20, 11, 9, 55, 29, 42, 6	8	—	—	—

4. Reversion pattern of *try D* and *C* mutants: The reversion pattern of twenty-three tryptophan requiring mutants induced in the presence of BUDR, AP and HNO_2 was investigated. Table 2 illustrates the response of these alleles. Fifteen, or 65%, of the twenty-three mutants were base analogue inducible; the rest were noninducible. The mutant alleles showed considerable specificity of response to the mutagens, and this is further emphasized if the relative amount of increased mutation is also taken into account. To illustrate this the data is presented in histograms (Fig. 4) which show that the base analogue inducible sites vary on their response to AP, BUDR and HNO_2. Some were highly inducible by AP and only a little by BUDR (*-33*, *-3*, *-70*), while others gave the opposite response (*-7*, *-66*) and still others responded about equally to both analogues (*-1*, *-10*, *-48*, *-35*, *-73*, *-79*, *-69*). The variations seem to indicate that the mutability of a given nucleotide pair is also dependent upon its position in the genome. The response of *D-79*, *-10* and *-78* is extremely interesting. The first two gave excellent results with AP and BUDR (and were chosen for kinetic studies – Rudner, 1961) but were completely unaffected by nitrous acid, while the latter acted in the reverse fashion. The apparent difference here seems to depend not on position in the genome but rather on the kind of base pair which is being altered. Of considerable interest is the mutagenic response of the three sets of alleles which according to

recombination tests are located on the same site (*tryD-10* and *-11*; *tryD-7* and *-66* and *tryD-29* and *-42*). The last set of mutants showed an identical response (5% increase) to the mutagens; *D-7* and *-66* differed with respect to treatment by AP and the degree of response to the others; *D-10* and *-11* were completely dissimilar, the former being base analogue inducible and the latter being base analogue noninducible. The pairs with a relatively similar response pattern are also similar in other respects: *-29* and *-42* act in the same fashion in recombination tests producing the same kind of aberrant results; *-7* and *-66* have an identical suppressor pattern; while the last pair, *-10* and *-11*, are also different with respect

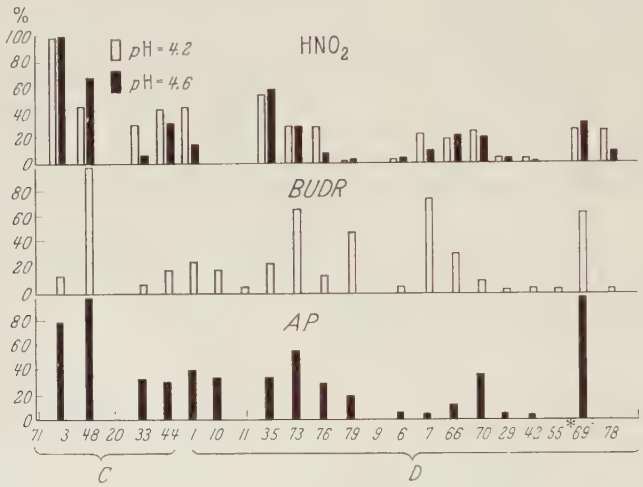


Fig. 4. The inducibility of the individual mutants of *tryD* and *-C* loci illustrated as per cent mutagenic response. The behavior of *tryC-48* is taken to constitute a 100 % response for AP-induced (frequency = 4.5×10^{-6}) and for BUDR (frequency = 1×10^{-6}) *try*$^+$ mutants. The response of *tryC-3* to HNO_2 is taken as 100 % inducibility (254 colonies per plate) in the presence of 1N $NaNO_2$ – acetate buffer at pH 4.2 and 4.6. + = *tryD-69* in the presence of AP. An increase in both the frequencies of reversion and suppressor mutation was observed upon plating on enriched minimal agar, but the same increase was also observed on unsupplemented agar. In the latter case, no distinction could be made between small and large colonies. Unlike what was usually observed (see Table 1) the number of colonies on all three types of minimal plates was always the same with a frequency of 6×10^{-6}. It was therefore not possible to determine the extent of increase in the number of revertants. * – *tryD-55* mutates to a great extent on the plates after long periods of incubation. This process occurs with or without treatment in the presence of all three mutagens. At the time of scoring (after 48 or 72 hours incubation at 37 °C) there was no significant effect of the treatments

to suppressibility; the suppression of the former does not affect the latter as determined by transduction tests (BALBINDER 1961). These differences make it possible to conclude with certainty that *tryD-10* and *-11*, though occupying the same map position, are due to two different mutations at the same site. While with the other pairs (especially *-20* and *-42*), the possibility that they are identical alleles still exists.

BALBINDER (1961) tested the response of these mutant strains to the alkylating agents diethylsulphate (DES) and beta-propiolactone (BPL). DES induced reversions in 10 of the 22 mutants tested (*D-79* was not analyzed), of which 14 also responded to base analogues and nitrous acid. It appears that any mutant that fails to react to DES treatment does not respond to treatment with any of the three mutagens discussed above. Although the alkylating agents react with many

compounds, there is evidence from studies with phages that their mutagenicity is a result of their reaction with DNA (Loveless 1959; Bautz and Freese 1960). These agents can directly alkylate the purine or pyrimidine ring resulting in methylated bases such as 7-methyladenine or 7-methylguanine (Reiner and Zamenhof 1957; Lawley 1957). It is therefore not surprising to observe a similarity between the mutagenic effect of DES and the effect of incorporated base analogues into DNA.

All twenty-three mutants analyzed were found capable of reverting spontaneously to prototrophy (Table 3). This led to the assumption that the original

Table 3. *Origin and spontaneous background of twenty-three alleles of tryD- and -C loci of Salmonella typhimurium in relation to their response to base analogues and* HNO_2[1]

Locus	Mutant No.	Origin	Frequency per 10^9			Base analogue inducible
			MM	Try±MM	enMM	
C-	71	AP	1.5	3.6	8.3	—
	3	UV	1.5	3.2	4.0	+
	48	sp	2.3	5.7	10.0	+
	20	sp	1.1	2.4	6.5	—
	33	sp (mut)	0.7	1.2	4.5	+
	44	sp	0.7	2.1	7.1	+
D-	1	UV	0.65	1.2	2.9	+
	10	sp	0.4	0.9	2.5	+
	11	sp	3.5	7.5	11.9	—
	35	sp	0.8	1.4	3.1	+
	76	AP	0.2	0.5	1.0	+
	73	AP	0.7	1.3	3.1	+
	79	AP	0.8	1.3	5.0	+
	9	sp	0.0	0.0	0.8	—
	6	sp	0.6	1.3	2.5	—
	7	UV	0.6	1.1	2.4	+
	66	sp (mut)	0.6	1.4	2.6	+
	70	sp (mut)	1.1	1.9	4.0	+
	29	sp (mut)	0.5	1.1	2.4	—
	42	sp	0.6	1.2	2.5	—
	55[2]	sp	5.0	7.5	22.5	—
	69	sp (mut)	2.6	4.8	7.1	+
	78	UV	1.1	2.4	3.1	+

[1] sp = spontaneous; UV = ultraviolet radiation; sp (mut) = spontaneous from *LT-7* containing mutator gene (see materials and methods).

[2] Rapid plate mutation.

mutagenic change was a transposition of nucleotides rather than a deletion. Of the fifteen mutants which arose spontaneously, 9, or 60%, were inducible with one or more of the mutagens; of the 8 mutants isolated with AP or U.V., 7 responded to base analogues (see Table 2 and Fig. 4). On the contrary, Freese (1959c) has found that only 14% spontaneous rII mutants and 2% proflavin induced mutants in the coliphage T 4 were base analogue inducible. Similar results were reported by Kirchner (1960) who used a series of alleles at the histidine locus in two strains of *Salmonella typhimurium*. All 87 mutants arose spontaneously; 46 were derived from the LT-7 strain in which the mutator gene occurs and 41 from LT-2 strain lacking mutator. The LT-7 auxotrophs with the mutator exhibited an increase in mutation to prototrophy with either or both

base analogues (AP and BUDR); the LT-7 strains without the mutator were unaffected by either base analogues. Of the 41 LT-2 strains tested, 27% showed an increase with either or both base analogues. The 11 of the 12 histidine requiring mutants that were obtained after treatment with AP showed an increase in reversion frequency with BUDR. The U.V.-induced auxotrophs on the other hand showed no particular mutational pattern. These results are consistent with the observations made by FREESE (1959c) who found that back mutation pattern of a given allele depended on the manner in which the forward mutation was induced. Mutants obtained originally with base analogues are easily reverted by them and consequently belong to one class referred to as "transitions". A fraction of the mutants which arose spontaneously or after U.V. treatment also belong to the transition class. There are differences among the various loci studies as to how large this fraction is (rII – 14%, histidine locus - 27%, *tryD* and *C* – 60%); it may represent a locus-specific phenomenon. The rest of the mutants which arose spontaneously or with U.V. and proflavin belong to a different class(es) of transpositions and are base analogue noninducible. They seem to have arisen through a fundamentally different type of chemical alteration (a "transversion"? FREESE 1959a) which cannot be reversed by base analogues but only by other agents.

II. The chemical incorporation of base analogues into DNA

1. The incorporation of BU into DNA of *try C 3*. The chemical incorporation of 5-halogenated uracils into the DNA of bacteria and viruses was first reported simultaneously by DUNN and SMITH (1954) and by ZAMENHOF and GRIBOFF (1954). The early chemical analyses were performed mostly with *E. coli* $15t^-$. When the strain was grown on basal medium the amount of incorporation was 18–28% in terms of thymine but there was evidence of considerable inhibition. When enriched medium was used the inhibition was minimized and the amount of incorporation increased to 48%. Base ratio analysis confirmed that the halogenated pyrimidines were in fact incorporated in the DNA. The relative proportions of adenine, guanine and cytosine remained unchanged. The amount of thymine however was decreased so that the relative proportion of thymine and 5-halogenated pyrimidine to the total bases was equal to that of thymine in normal nucleic acid. Incorporation of 5-bromouracil in the DNA of wild type strains appeared negligible. This was increased with the use of the deoxyribosylated analogue or by interference with the endogenous methylation of deoxyuridylic acid by aminopterin or sulfanilamide (ZAMENHOF *et al.* 1958; DUNN and SMITH 1957).

The mutant strain *C-3* was selected for these studies and cultured on enriched medium supplemented with BU and aminopterin (200 μg/ml of each). Since the procedure followed was that described by ZAMENHOF *et al.* (1956) for the *E. coli* thymine-requiring mutant, this strain ($15t^-$) was grown in the presence of 100 μg BU/ml, and then analyzed for BU incorporation into DNA as a control for the procedure. The cells were harvested after 18 hours incubation with aeration at 37° C, whereupon from 65–70% were viable. Highly polymerized DNA was isolated after lysis with Duponol employing salt extraction and alcohol precipitation. Estimation of the nitrogenous bases was made on a perchloric acid hydroly-

sate, followed by a two-step separation by paper chromatography. The first solvent, n-butanol saturated with water, separated thymine and BU from the slower components ($R_f = 0.47$). The band was cut out, extracted and rechromatographed with n-butanol-NH_3 as the solvent whereupon the BU spots clearly separated from the faster thymine. Table 4 shows the values obtained. This

Table 4. *The incorporation of 5-bromouracil (BU) into the DNA of Escherichia coli 15t⁻ and Salmonella typhimurium try C-3*

	15 t⁻		*try C-3*			
	Thymine	BU	Thymine	BU	Thymine	BU
	Preparation I		Preparation I		Preparation II	
R_f values in n-butanol-NH_3	0.34	0.21	0.34	0.20	0.33	0.19
μmole recovered	0.550	0.381	1.438	0.602	0.183	0.091
% incorporation (BU/BU + T) . .	40.9		29.5		33.2	

adequately demonstrated that *Salmonella typhimurium* just as *E. coli* incorporates BU into its DNA. Since a number of investigators have shown that BU can be incorporated into the DNA of several species of bacteria (Dunn and Smith 1957; Zamenhof *et al.* 1956a; Wacker *et al.* 1954) and because in every instance the analogue appeared to be quantitatively replacing only thymine residues, it was therefore deduced that BU replaced thymine specifically in the DNA of *Salmonella typhimurium* even though base ratio analysis was not performed here. This kind of chemical analysis was not done for other growth conditions, such as the use of basal medium supplemented with BUDR, aminopterin and metabolites or in the presence of FUDR. Other criteria such as viability, mutagenesis or sensitivity to U.V. light (Greer 1960; Lorkiewicz and Szybalski 1960) are usually indicative of incorporation.

2. The chemical incorporation of AP into DNA. Prior to these chemical studies, biological data pointed to incorporation of AP as the cause of mutagenesis rather than to the possibility that AP unbalanced some nucleic acid precursor pool which then led to the incorporation of a normally synthesized base at an unusual site (Rudner 1960, 1961). To date, the literature on the chemistry of AP is extremely sparse. Freese (1959b) speculated that incorporation of AP into NNA was structurally possible although experiments showed it to replace $< 1\%$ of adenine, if any. The only report is a recent publication by Wacker *et al.* (1960) who claim that *E. coli B* cells transform AP into adenine and guanine while a very small amount of unchanged AP is found in DNA and RNA. They used tritiated AP and analyzed the nucleic acids after a perchloric acid hydrolysis. They failed to mention whether this conversion was a complete decomposition of AP to small fragments, followed by reutilization for the biosynthesis of purines (and possibly pyrimidines), or whether it was a direct enzymatic conversion. Furthermore, AP is heat labile (see later) and the acid-heat hydrolysis employed in their analysis could be the cause for such a low recovery of unaltered incorporated AP.

α) Growth conditions. It became evident from preliminary studies that although AP was incorporated into DNA, it was to a much smaller extent than BU into DNA or azaguanine into RNA (Matthews 1953). To enhance the incorporation of AP, purine-requiring mutants or antimetabolites that inhibit the biosynthesis

of purines should be used. STRELZOFF (1960) has devised a method for following the incorporation of a given base analogue into DNA. Essentially, it involves observing the increase of DNA as purineless or pyrimidineless mutants are grown on limiting amounts of growth factor and on excess of base analogue. If the cells synthesize more DNA than the control population grown only on limiting amounts of growth factor, one can assume two possibilities: (1) the analogue is incorporated into DNA and/or (2) the analogue is converted to another component normally built into DNA. Base ratio determinations and tracer experiments would then assist in distinguishing between the two possibilities. Following this outline a variety of purine, adenine and guanine-requiring mutants were grown in the presence of limiting amounts of growth factor (10 μg/ml) and an excess of AP (50, 100, 150 and 200 μg/ml). The cells were incubated at 37° C and aliquots were withdrawn from time to time for the determination of DNA per ml culture. All the adenineless and the single guanine requiring mutants analyzed gave negative results; no net increase of DNA above that of the controls was observed. However, two out of the four purine requiring mutants, *pur-107* and *pur-109*, gave positive results. Although the results were not as dramatic as obtained with a thymine-requiring mutant in the presence of BU (STRELZOFF 1960), a 50% net increase of DNA was demonstrated after 40–45 hours incubation (Fig. 5). There was a considerable drop in viability under these conditions with many cells forming long filaments or "snakes". This phenomenon has been described for *E. coli 15t⁻* grown in the presence of BU (ZAMENHOF et *al.* 1956b; DUNN and SMITH 1957). Interestingly enough, the amount of unaltered AP which was recovered from the DNA was much smaller (Max. 1.0–1.3% of total DNA) than that required to account for a net increase of 50%. It is possible that the extra DNA synthesis was due to the transformation of AP into adenine and guanine (WACKER *et al.* 1960).

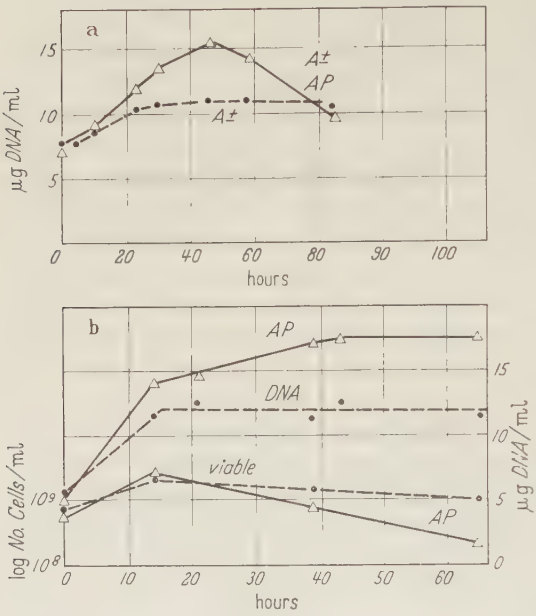


Fig. 5. a The increase of DNA synthesis in the presence of limiting amounts of adenine (10 μg/ml) and excess amounts of AP (50 μg/ml) in the mutant strain *pur-107*. DNA was determined colorimetrically using Dische diphenylamine reagent on the hot 5%-TCA soluble fraction. The extra DNA synthesized in the presence of AP began to degrade after 50 hours. ● = control population; △ = experimental population. b The viability of mutant strain *pur-109* and the increase of DNA synthesis in the presence of limiting amounts of adenine and 100 μg AP/ml. ● = control population; △ = experimental population. The number of viable cells was determined by plating appropriate dilutions onto nutrient agar plates supplemented with 40 μg adenine/ml

A second growth condition, the H-medium described by FREESE (1959b), proved to be superior. The addition of histidine and thiamine to the growth

medium reduce the amount of adenylic acid (AMP) that enters the histidine pathway (Magasanik 1959). Exogenous adenine which is converted to AMP participates actively in the synthesis of inosinic acid (IMP) the precursor of all other purines in the following manner:

(a) AMP → IMP → XMP → GMP[1]
(b_1) AMP → PRPP → Imidazole glycerol — P + AICAR
(b_2) AICAR → IMP → XMP → GMP

Freese found that when two purine-requiring mutants of *E. coli*, *B-96* and *B-97* (the first is blocked in the conversion of AICAR → IMP and the second in the enzyme which removes succinic acid from purine in the *de novo* synthesis of AICAR and in the conversion of IMP → AMP), are grown in H-medium supplemented with 500 μg AP/ml, *B-96* cells develop into threads uo to 0.1 mm long (97% are inviable), while *B-97* cells divide normally after some transitory lengthening. The difference between the two strains, according to Freese, is that, when they are grown in adenine, *B-97* can make IMP from AMP by the two pathways mentioned above, while *B-96* can do so only by the deamination of AMP → IMP. The different effects of AP upon these two bacteria suggested to Freese that AP interferes with the deamination of AMP → IMP (reaction a). There was indeed less filament formation when *B-96* was grown with AP and Xanthine, hypoxanthine or guanine. Some of these observations were confirmed in *Salmonella* with *pur-107* mutant strain grown on H-medium. After 16–18 hours incubation the majority of the cells developed into long threads and 72% were unable to proliferate. Further, the frequency of reversions increased from a spontaneous background of 10^{-10} to 3.7×10^{-7} in the presence of AP (Fig. 6).

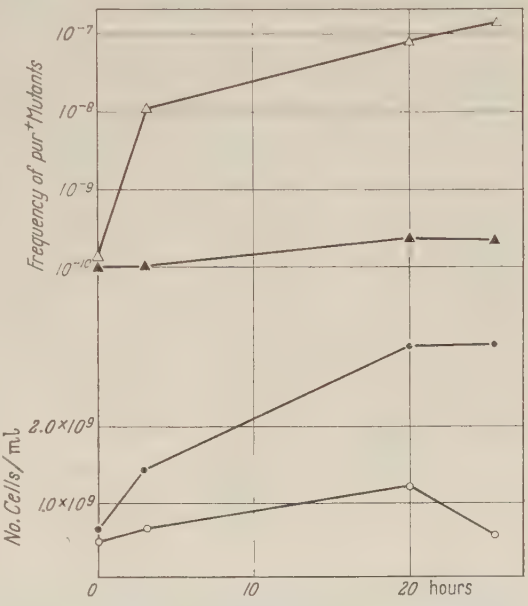


Fig. 6. Viability and the increase in back mutation frequency of *pur-107* grown in the presence of 500 μg AP, 40 μg adenine, 10 μg histidine and 0.25 μg thiamine/ml. The number of viable cells was determined on nutrient agar supplemented with 40 μg adenine/ml (● = control; ○ = experimental). Mutants (*pur*$^+$) were determined on MM plates (▲ = control; △ = experimental). The majority of the bacteria grown in the presence of AP developed into long threads

β) The chemical properties of A P. The presence of AP in any given preparation is demonstrated without difficulty because of its strong fluorescence in an acid or alkaline solution. Although guanine compounds also fluoresce strongly, they do so only in an acid solution. AP also possesses relatively low U.V. extinction values in either acid or base (pH 2-$\mathscr{E}$max 314 mμ = 4.0; pH 12-$\mathscr{E}$max 303 mμ = 5.8).

[1] XMP = Xanthosine monophosphate, GMP = guanylic acid, PRPP = 5-phosphoribosyl-pyrophosphate, AICAR = 5-amino-4-imidazolecarboxylic acid ribotide.

With a suitable filter an AP spot exposed to a U.V. lamp can be seen to give off a bright blue fluorescent light; this property is greatly enhanced at high pH's which can be obtained by placing developed papers in atmospheric NH_3. A fluoroscope sheet placed over the spot eliminates the fluorescent light leaving a dark but much smaller spot.

When AP was autoclaved or treated in 7.5N PCA at 100° C for 1 hour it was found to behave as if two substances (I and II) were present and resolvable by paper chromatography (Table 5). AP-I reacted identically to untreated AP, while

Table 5. *The R_f values of purines and pyrimidines bases or 5'-deoxynucleotides in relation to 2-aminopurine untreated or treated in 7.5 N PCA at 100° C for 1 hour.* The values for isobutyric acid-NH_4OH are relative to deoxyadenylic and taken as 100. DR = deoxyribotide

Bases	n-butanol-NH_3	Isopropyl-HCl	Deoxynucleotides	Isobutyric acid-NH_4OH	Isopropyl-HCl[1]
Guanine	0.16	0.23	Guanine-DR	52	
Adenine	0.38	0.32	Adenine-DR	100	—
Cytosine	0.25	0.44	Cytosine-DR	78	0.60
Thymine	0.49	0.76	Thymine-DR	62	0.81
2-aminopurine	0.33	0.19	2-aminopurine-DR	87	—
After treatment					
2-aminopurine I	0.33	0.19			
2-aminopurine II	0.10	(0.30)[2]			

[1] In isopropyl-HCl the purines are no longer deoxyribotides due to the hydrolysis of the N-glycoside bond in acid.

[2] Not always observed due to the closeness of the spot to adenine and guanine.

AP-II fluoresced but failed to absort any U.V. light. The probable explanation lies in the compounds very low extinction value so that in the given concentration it was impossible to measure its absorption. Comparative U.V. absorption studies showed that the absorption curve for heat-acid treated AP appeared distorted with an increase at the range of minimal absorbance (260–270 mμ) and a decrease at the range of maximal absorbance (310–320 mμ). This suggests a conversion of AP to another purine possibly through a deamination of the 2-amino group.

On the basis of these observations, it was evident that the standard perchloric acid hydrolysis was unsuitable when one sought to measure the incorporation of AP. The DNA was therefore hydrolyzed by the use of enzymes or mild acid treatment.

γ) Incorporation studies. The mutant strain *pur-107* was cultured under the second growth condition in the presence of 500 μg AP/ml. DNA was then extracted by the method described earlier. Digestion with DNase and snake venom diesterase followed to yield 5'-deoxymononucleotides. The next procedure served to establish the efficiency of the enzymatic digestion and the presence of AP in nucleotide form. This was done by paper chromatography with water saturated n-butanol as the solvent and ammonium bicarbonate added to the solvent and placed at the bottom of the tank. In this solvent mononucleotides do not travel and indeed none of the reaction mixture, including AP, came down. It was therefore concluded that the AP had been incorporated into the DNA and was in a deoxynucleotide

form (APDR). The R_f of APDR was determined with the solvent crossout isobutyric acid-NH_4OH. It fell between cytosine-DR and adenine-DR, while free base (commercial AP) used as control appeared very close to the solvent front (Table 5). This solvent system proved unsuitable for quantitative determinations. The solvent of choice was isopropyl-HCl-H_2O; with it one can spot the paper heavily with the enzymatic hydrolysate and still obtain good separation between the pyrimidine deoxyribotides and the purines which have become free bases due to the hydrolysis of the N-glycosidic bond in the presence of HCl. In this solvent, AP migrated very close to guanine. AP was then isolated and rechromatographed with butanol-NH_3 as the solvent.

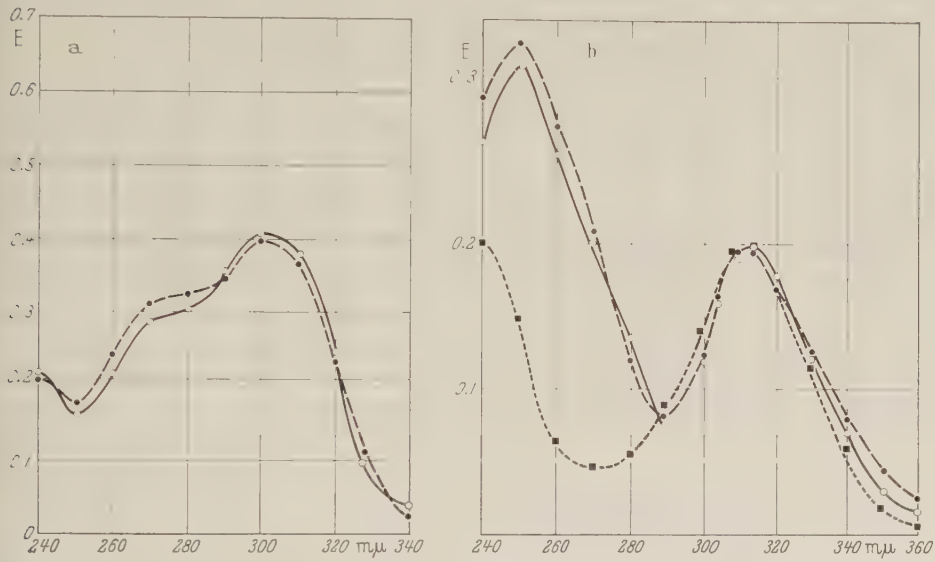


Fig. 7. a Ultraviolet-absorption at pH 13 (0.1 N NaOH) of 2-aminopurine (AP) isolated from *Salmonella typhimurium pur-107* DNA as compared with known chromatographed AP. The control spectrum is corrected to give the same value of E at 300 mμ. Experimental AP = ●; Control AP = ○. b Ultraviolet-absorption spectra at pH 1 (0.1 N HCl) of control and experimental AP. The control spectrum is corrected to give the same value of E at 314 mμ. Experimental AP = ●; known chromatographed AP = ○; known unchromatographed AP = ■

The last criterion used for the characterization of the compound, which appeared to be AP on the basis of its R_f mobility and fluorescence, was its ultraviolet absorption in acid and base. Since it has a low extinction value and the amount incorporated was relatively small, a number of heavily spotted papers were pooled. The absorption curves of both known AP and the incorporated one isolated from DNA are presented in Fig. 7. In 0.1 N NaOH (pH 12) the absorption curves were identical (with a minimum absorption at 250 mμ and a peak at 303 mμ). On the other hand, in acid (0.1 N HCl) the known and the experimental compound were different from the unchromatographed AP at the lower wave lengths. The unchromatographed AP had a rather broad range of minimal absorbance around 274 mμ, while the two chromatographed samples had a narrow minimum which shifted to 290 mμ. In addition, a second maximum at 250 mμ was evident; the position of the peak at 314 mμ was unchanged (the shift

might be due to changes which take place in acid). It was therefore concluded that the compound incorporated into DNA of *pur-107* was unaltered AP.

The quantitative analyses of two DNA preparations obtained from *pur-107* grown in H-medium and one from a control grown under the same conditions without AP are presented in Table 6. About 1% AP of total DNA was recovered in both preparations. The data suggest that both adenine and guanine can be

Table 6. *The incorporation of 2-aminopurine (AP) into the DNA of Salmonella typhymurium pur-107 grown in H-medium*

An enzymatic digest of DNA (*ca.* 1.5 mg for I and *ca.* 3.0 mg for II) was spotted and developed in isopropyl-HCl. The pyrimidines were still in the form of deoxyribotides while the purines including AP became free bases due to the hydrolysis of the N-glycosidic bond in acid. % = moles/100 moles of total recovered bases. DR = 5′-deoxyribotide.

	Control		Experimental			
	I		I		II	
	μmole	%	μmole	%	μmole	%
Thymine DR . . .	0.955	23.1	0.926	22.9	1.935	22.9
Adenine	0.953	23.0	0.911	22.5	1.930	22.8
Cytosine DR . . .	1.112	26.9	1.097	27.1	2.285	27.0
Guanine	1.118	27.0	1.072	26.5	2.225	26.3
2-aminopurine . . .	—	0.0	0.051	1.2	0.091	1.1

replaced by AP. Since the values recovered are relatively small, one can only speculate on the per cent replacement of both purines. The data for this particular mutant strain further suggest that AP has a preference in replacing guanine over adenine. The latter is indicated from another analysis where the purines were removed from DNA in the production of apurinic acid. The mutant strain *pur-107* was grown in the presence of a limiting amount of adenine (10 μg/ml) and 500 μg AP/ml. Apurinic acid produced by a mild acid treatment. Table 7 presents the amounts of free purines recovered after the treatment. The theoretical ratio of guanine/adenine is 1.17; the control ratio was only 1.08, indicating a low recovery of guanine. Nevertheless, the ratio G/A in the experimental group was even lower. When the amount of AP is added to the amount of guanine the ratio becomes much closer to the control. WACKER *et al.* (1960), who cultured bacteria in the presence of tritiated AP, found that the specific activity of guanine isolated from their DNA was 2—3 times higher than that of adenine, indicating that AP was converted to guanine more frequently than to adenine.

Table 7. *The amounts of free purines including 2-aminopurine (AP) recovered from BNA of Salmonella typhimurium pur-107 after mild acid hydrolysis (0.1 N HCl at 37°C for 18 hours)*

The mutant strain was grown in limiting amounts of adenine (10 μg/ml) and excess of AP (200 μg/ml).

	Control I	Experimental	
		I	II
	μmole	μmole	μmole
Adenine (A).	2.167	1.424	1.710
Guanine (G)	2.332	1.413	1.743
2-aminopurine (AP) . .	—	0.076	0.083
G/A	1.08	0.99	1.02
G+AP/A	—	1.06	1.07

Despite these inconclusive chemical considerations, it was possible to conclude from mutational analysis alone that AP most frequently replaces adenine which it resembles structurally (Rudner 1961).

D. Discussion

The effectiveness of an analogue in replacing a normal component in nucleic acid depends upon its structural features and on the metabolism of the cell it enters. A base analogue should be sufficiently like the normal base to be incorporated into DNA, but different enough to cause a genetic change. The Watson and Crick model imposes certain restrictions on the types of purines and pyrimidines which may become incorporated into the DNA structure. Positions 1 and 6 or 2 in the rings of the bases must have the configuration necessary to form the hydrogen bonds linking the pairs of bases in the two chains of the double helix. Also, the DNA with a base analogue has to fulfill Chargaff's (1955) regularities; that is, there must be a stoichiometric equality between the sum of the purines and the sum of the pyrimidines and the content of 6-amino groups must equal the content of 6-keto groups. These regularities are, however, for the entire normal molecule of DNA; minute distortions probably can take place which are not easily detected. To date, only one DNA has been isolated which presents a structural problem. Dunn and Smith (1958) report that a large amount of 6-methyladenine has been found in DNA isolated from *E. coli 15t*$^-$. This analogue substitutes for a portion of thymine (although it is an adenine analogue) in the DNA of that organism. This DNA cannot be brought into accord with the Watson and Crick model and does not fulfill Chargaff's regularities; hence it is supposed to be abnormal.

Structurally, 2-aminopurine resembles both adenine and guanine, however, pairing by means of two hydrogen bonds in its normal amino state is only possible with thymine residues. On the other hand, when AP is in its rare imino state after a tautomeric shift, it can pair with cytosine. Freese (1959b) has pointed out that AP can form one hydrogen bond to cytosine (between the 2-amino of AP and the 2-keto of cytosine) even when both bases are in their normal amino state. This one bond alone, since no other groups introduce steric hindrance, may be sufficient to cause frequent pairing of AP with cytosine. Since AP can pair with both cytosine and thymine in its normal amino state or rare imino state, it might therefore be expected that pairing errors of AP would be more frequent than those of BU. If AP can pair so easily with both pyrimidines in its normal amino state, one might ask why is it that such a small amount becomes incorporated into DNA? The answer probably lies in the enzyme system which converts AP into a direct DNA precursor. Many purine and pyrimidine nucleotides may be formed directly by the enzymatic condensation of the free base and 5-phosphoribosyl pyrophosphate (PRPP); the enzyme responsible for this reaction has been named 5-phosphoribose pyrophosphokinase (Khorana *et al.* 1958). Furthermore, the conversion of ribonucleotides to deoxy compounds has been shown in a variety of biological systems (Rose and Schweigert 1953; Edmonds 1958). The conversion of base analogues to nucleotides *in vivo* has been reported for 8-azaguanine, 2,6-diaminopurine and 6-mercaptopurine (Way and Parks 1958). This enzyme system most probably is capable of converting AP into a deoxyribotide. On the

basis of WACKER's report and on the data presented here, one can visualize the following events. AP molecules penetrate the bacterial cell with no difficulty; once in the soluble pool the majority are converted into guanine and adenine with a very small amount transformed to deoxyribotides and ribotides which in turn are built into DNA and RNA. A competition probably exists between the enzyme system which transforms AP into normal free bases and the system which converts AP into a nucleotide. This competition appears to be greatly enhanced when a purine requiring organism is used.

The replacement of the methyl group of thymine by bromine, which has approximately the same van der Waals radius, may explain why BU is incorporated in large quantities. It is interesting that studies with bacterial polymerase, using the triphosphate of BU-deoxyribose, have shown that the enzymatic reaction is governed by the specific base pairing rule proposed by WATSON and CRICK. The analogue substituted for thymidine 5′-triphosphate but not for any of the other three precursors, ATP, CPT or GTP (BESSMAN *et al.* 1958). In order for BU to pair erroneously with guanine, it has to be in its rare enol state. MESELSON (in FREESE 1959b) has speculated that the higher electronegativity of bromine as compared to the methyl group should increase the frequency of tautomeric shifts in BU over that in thymine. LITMAN and PARDEE (1956b) have assumed that BU is more stable in the enol form than is thymine. Although at the present time there is no evidence to substantiate either of these assumptions, nonetheless, the ability of BU to pair with guanine as judged by the degree of mutagenicity in various bacterial system is not very striking. Furthermore, the abundance of BU molecules introduced into DNA results in cell death. This is probably due either to the production of lethal mutants or to the fact that the genetic code ceases to convey any specificity. The latter possibility is supported by the recent findings of SHAPIRO and CHARGAFF (1960) who reported that when BU is incorporated into the DNA of thymineless bacteria a complete reorganization and distortion of the nucleotide sequence pattern takes place. It seems reasonable to assume that a viable mutant is induced in cells that have incorporated relatively small amount of BU.

Since these mutagenic studies were performed *in vivo*, and not *in vitro* on transforming principle or on the RNA of TMV, as can be done with nitrous acid, it can still be argued that the mutagenic action of BU or AP is not by direct incorporation into DNA but is rather indirectly by unbalancing nucleic acid metabolism. The fact that many base analogues which do not seem to be incorporated into DNA inhibit the metabolism of nucleic acid and yet are not highly mutagenic makes it probable that the effect of BU and AP is a direct one. It would still have to be demonstrated experimentally that the induced mutations resulted from the misdirection of DNA replication by incorporated AP or BU. This demonstration will most probably have to be made in *in vitro* systems such as transforming principle or in the Kornberg system, provided that the newly synthesized DNA can be first shown to have biological specificity. In the accompanying paper, biological data will be presented in an effort to demonstrate that BU and AP mutagenesis are mediated by their incorporation into DNA, and that incorporation *per se* is insufficient for mutation. Rather, mutations are induced by errors in base pairings during replication of DNA. Whether the error is made at

the time of incorporation, or subsequently, the new stable base pair at the mutant site is established only when two replications of DNA succeed incorporation.

Summary

Reversion patterns of twenty-three mutants at two tryptophan loci (*tryC* and *D*) in *Salmonella typhimurium* have been investigated after treatment with three specific mutagens. The compounds used were the base analogues, 5-bromouracil (BU) and 2-aminopurine (AP), and nitrous acid. The mutant alleles showed considerable specificity of response to the mutagens. The increased frequency of mutants after growth in the presence of base analogues was not due to selection of pre-existing mutants as shown by reconstruction experiments. It was determined that BU can be incorporated into the DNA of this organism by replacing thymine. A small amount of unaltered AP was recovered from the DNA of a purine-requiring mutant. These findings suggest that BU and AP induce "base-pair transitons" in *Salmonella typhimurium* as had been previously observed for bacteriophage (Freese 1959).

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MUTATION AS AN ERROR IN BASE PAIRING

II. KINETICS OF 5-BROMODEOXYURIDINE AND 2-AMINOPURINE-INDUCED MUTAGENESIS*

By

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With 10 Figures in the Text

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A. Introduction

Base analogues when incorporated into DNA may exert a mutagenic effect by causing the formation of a "transition", namely the replacement of purine by purine or pyrimidine by pyrimidine (A:T ⇌ G:C)[1], thus altering the base sequence of DNA (Freese 1959b). During or following the incorporation of some base analogues there is an increased likelihood of error in base pairing. The new stable pair at the mutant site is established only when two replications of DNA succeed the initial incorporation (Fig. 1). A similar model for spontaneous mutations was originally proposed by Watson and Crick (1953) who suggested that the nitrogenous bases may sometimes be in a rare tautomeric state. For example, if the 6-keto group of a thymine residue changes transitorily to the enol form, then thymine would no longer be capable of pairing with adenine but would instead pair with guanine which would then replace adenine in the new complementary chain. In a subsequent replication the guanine residue would pair with cytosine and a transitional change could be accomplished. Thus the process of base analogue mutagenesis consists of at least two step. First, the incorporation (or formation *in situ*) of an unusual base in one strand of the double helix of DNA, and second, the replication of this altered chain in order to establish a modified base sequence. The first event can be considered the registration of a potential mutant; the second — the fixation event. The mutated allele has still to change the biochemical pattern of the cell for a clone with a new phenotype to appear.

There are two distinct possibilities by which base-pair transitions can occur in replicating DNA. These can come about by "errors in replication" or by "errors in incorporation" (Freese 1959b). Essentially, the difference between them is seen in (1) the direction of the transitional change, (2) the DNA replication cycle during which the erroneous pairing takes place, and (3) the way in which the mutants increase (Fig. 1, 9). It is important to emphasize that, whether the error in base pairing occurs in incorporation or in replication, the new stable pair at the mutant site is established only when two replications of DNA succeed incorporation.

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[1] The following abbreviations are used in this paper: A – adenine, G – guanine, C – cytosine, T – thymine, BUDR – 5-bromodeoxyuridine, AP – 2-aminopurine.

Experiments were designed to test the model of mutation by transitional change with the following considerations in mind. 1. Do the mutations induced by BUDR and AP occur concurrently with DNA synthesis? 2. How many replications of DNA are necessary to obtain a heritable change in genotype and, furthermore, does the time at which the mutant phenotype develops coincide with the time of establishment of the new genotype? 3. Can a mutant still develop even though the mutagen has been removed after the first DNA replication? 4. Is it possible to distinguish between the two types of errors induced by these base analogues thereby identifying the base pair at the mutant site? These studies were performed with populations of tryptophan-requiring bacteria whose division had been synchronized by fractional filtration (MARUYAMA and YANAGITA 1956). The findings are presented in a manner whereby, in the kinetic sections the experiments with each mutagen are analyzed in terms of the first three considerations. The third section will deal with a statistical analysis of the increase in mutant numbers observed for both mutagens in an attempt to distinguish between the two types of errors.

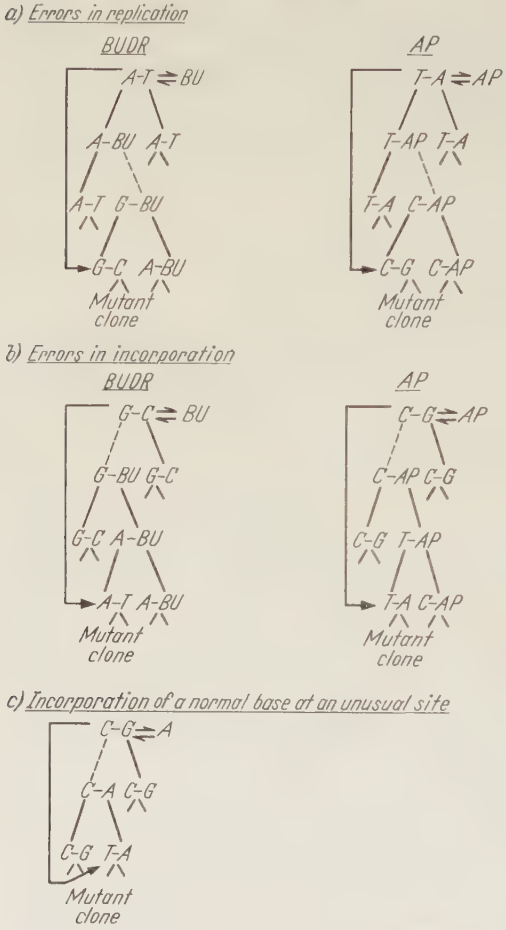


Fig. 1a—c. Models of mutation by transitional changes induced with base analogues. Transitions can occur either as (a) errors in replication or (b) errors in incorporation. Furthermore, they can occur as a result of the incorporation of a normal base at an unusual site (c). The model is based on the assumption that AP is an adenine analogue and can pair erroneously with cytosine while BU is a thymine analogue and can pair erroneously with guanine. The broken lines illustrate these rare events. Mutant clones are established two generations after the incorporation of base analogues (a), (b), and one generation after the incorporation of a normal base at an unusual site (c)

B. Materials and Methods

The materials and methods described in the preceding paper (RUDNER 1961) were applied in the present work.

I. Bacterial strains

Two tryptophan requiring mutants of *Salmonella typhimurium LT-2* were employed for the kinetic studies. The mutant strains *tryD-79*, isolated in the presence of AP, and *tryD-10*, of spontaneous origin, were used for AP and BUDR-induced mutagenesis. Both responded to either mutagen but not to nitrous acid.

Four additional mutant strains *D-73*, *D-7*, *C-3*, *C-48* were employed for the statistical treatment of the mutant increase patterns (for their origin see Table 3 and RUDNER 1961).

II. Synchronization technique

Synchronization was achieved by a fractional filtration which is essentially MARUYAMA and YANAGITA's (1956) method. The principle of the technique is mechanical separation of smaller and larger cells by filtration of a heavy bacterial suspension through layers of filter paper. Twenty sheets of Toyo filter paper No. 126, together with one Toyo No. 1 on the bottom and two on the top, were placed in a special apparatus and were wetted with synthetic medium. 5 ml. of a suspension containing *ca.* $5-10\times10^{10}$ cells/ml were absorbed on the top. The pile of papers was then tightened with the inner screw ring of the apparatus. Vacuum filtration was carried out while 5.0 ml. aliquots of synthetic medium were continually applied at the top of the filter paper pile; eventually turbidity appeared in the filtrate. Only the small-cell fraction which passes through the pile into the filtrate was selected for these studies. The procedure was performed at room temperature using prewarmed synthetic medium. The time required for filtration was less than 5 minutes. The small-cell fraction was diluted in prewarmed synthetic medium to contain *ca.* 1×10^9 cells/ml. and incubated at 37° C with aeration. Aliquots were taken from the culture every 5 or 10 minutes. They consisted of two groups: (a) Two samples of 9 ml. of culture were placed into centrifuge tubes containing 1 ml. chilled 50% trichloroacetic acid (TCA); all these samples for nucleic acid analysis were frozen until analyzed together at the end of the experiment. (b) 5 ml. of culture were placed into another centrifuge tube which again was chilled; this sample was used for the determination of the number of viable bacteria and of mutants per ml. culture. Plating for viable counts was performed within 1–2 minutes after the removal of the sample by spreading appropriate dilutions onto nutrient agar. The remainder of the sample was washed twice in saline, concentrated to contain 5×10^8 cells/0.1 ml. and spread onto the various minimal plates (MM, Try$^+$MM and enMM, see RUDNER 1961).

III. Biochemical determinations

DNA was determined colorimetrically on samples which were precipitated with 5% TCA and washed once with cold 5% TCA. The nucleic acids were extracted by a modified SCHMIDT-THANNHAUSER (1945) procedure. The hot TCA-soluble fraction was extracted twice with 5% TCA at 100° C for 30 minutes. The DISCHE (1955) diphenylamine reagent was used to determine DNA colorimetrically at 595 mμ against a commercial sperm DNA (National Biochemical Corp.) solution as a standard.

C. Experimental

I. The time and mode of appearance of BUDR-induced mutants

The mutant strain *tryD-79* was employed for these studies. It has the advantage of a low background of spontaneous *try*$^+$ revertants; its response to BUDR is relatively high and no suppressor mutations are known to occur. Bacteria in a heavy suspension were pretreated for 18 hours in 500 μg aminopterin/ml. The purpose of the pretreatment was to render the cells thymine deficient prior to the addition of the mutagen, and to induce synchrony upon addition of BUDR or thymidine as had been found for *E. coli 15t*$^-$ (BARNER and COHEN 1956; STRELZOFF 1960). The pretreatment with aminopterin was bacteriostatic and indeed induced some phasing (Fig. 2a); with *E. coli*, on the other hand, the treatment causes a rapid loss of viability (OKADA, YAMAGISAWA and RYAN 1960). The cells were then incubated with 250 μg BUDR/ml., a mixture of essential metabolites and 250 μg aminopterin/ml. Aliquots were taken every 30 or 60 minutes for the determination of the number of viable parents and mutants and of DNA per ml. culture.

Mutation from auxotrophy to prototrophy occurred in accordance with DNA replication (Fig. 2b). Even though the cells began to die due to the incorporation of BUDR, mutants on the supplemented minimal plates Try$^+$MM) began to

appear after the first replication of DNA. Stable *try*$^+$ mutants appeared on unsupplemented plates after three DNA replications; two of these had occurred in liquid culture, the third on the plates. The third DNA replication, similar to that which takes place on the minimal plate, was observed when *try*$^-$ cells were placed in liquid medium devoid of tryptophan; there they increased their DNA to 88 per cent. This residual DNA synthesis has also been reported by MAALØE (1960) who observed it in his experiments on the transitions of bacteria between two steady states of growth. The amount of residual DNA synthesis after transfer

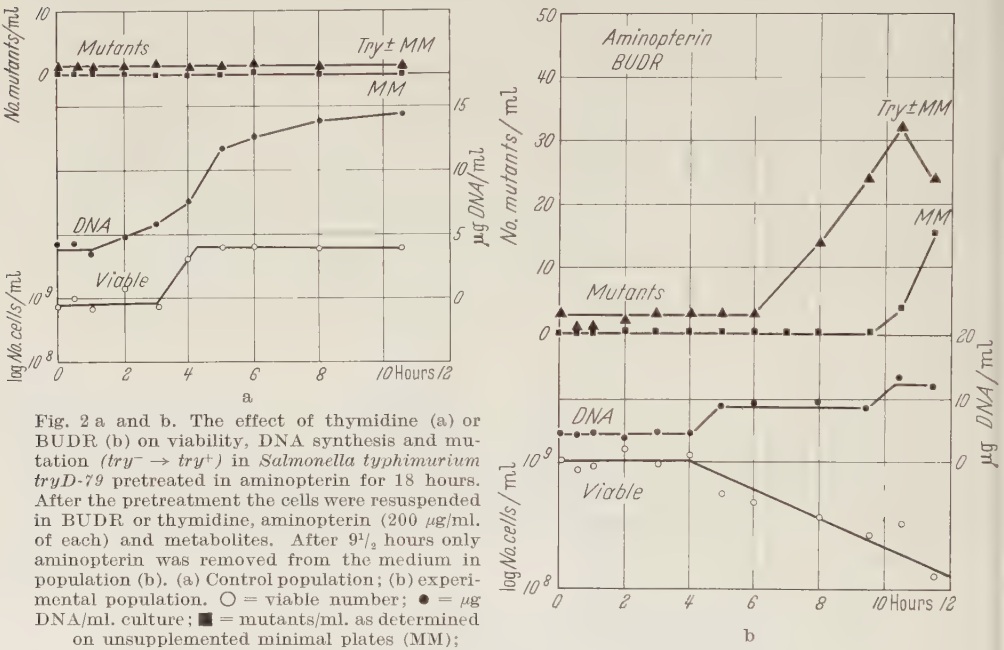


Fig. 2 a and b. The effect of thymidine (a) or BUDR (b) on viability, DNA synthesis and mutation (*try*$^-$ → *try*$^+$) in *Salmonella typhimurium tryD-79* pretreated in aminopterin for 18 hours. After the pretreatment the cells were resuspended in BUDR or thymidine, aminopterin (200 μg/ml. of each) and metabolites. After 9½ hours only aminopterin was removed from the medium in population (b). (a) Control population; (b) experimental population. ○ = viable number; ● = μg DNA/ml. culture; ■ = mutants/ml. as determined on unsupplemented minimal plates (MM); ▲ = mutants/ml. as determined on tryptophan enriched minimal plates (Try±MM)

of cells to media devoid of growth factor varies from 30—80%, depending on the state of the cells. It is assumed that the cells previously grown in the presence of the required growth factor have the enzyme system(s) and precursors to allow the completion of almost one replication.

Under other conditions, where mutagenesis was obtained without lose of viability, the culture was synchronized by fractional filtration and the small cell fraction was incubated with 10 μg FUDR/ml., 200 μg BUDR/ml. and metabolites or 0.2% Bacto vitamin-free casamino acids. The same picture emerged; stable tryptophan mutants appeared after a total of two replications in liquid, plus the additional replication on agar (Fig. 3). The removal of the cells from the BUDR containing medium after one DNA replication and resuspension in mutagen free media still allowed the appearance of mutants on unsupplemented minimal agar (Fig. 4, 10). The same occurred when the cells were allowed to increase their DNA to 30% in the presence of BUDR. The frequency of *try*$^+$ mutants at the end of the second replication in the fresh medium was about one third of that

observed in the first experiment where the cells were allowed to complete a full DNA replication in the presence of BUDR. This is consistent with the model of mutation by transitional change which predicts that after the base analogue has been incorporated, the further development of the change is independent of its presence or absence in the medium. Of course the mere removal of BUDR from the medium does not necessarily constitute its removal from the cells' nucleotide pool. However, the amount of acid soluble materials (amino acids and nucleotides) during logarithmic growth is probably too small to account for the mutagenesis observed (MANDLESTAM 1958; LARK 1960). A more direct

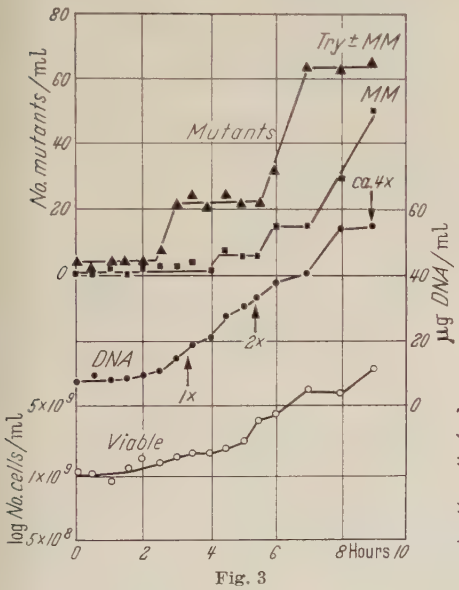


Fig. 3

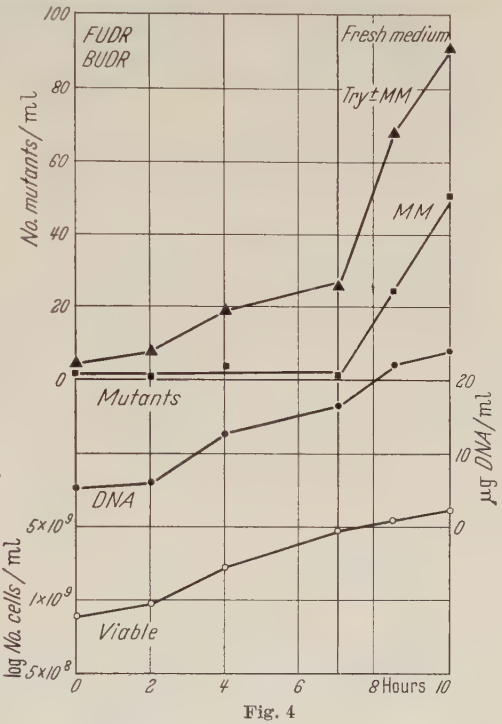


Fig. 4

Fig. 3. Time and mode of appearance of BUDR-induced mutants (*try*$^-$ → *try*$^+$) of *Salmonella typhimurium* strain *tryD-79*. The small cell fraction obtained by filtration was resuspended in 200 µg BUDR/ml., 10 µg FUDR/ml. and 0.2 % Bacto vitamin-free casamino acids. Stable *try*$^+$ mutants appeared on unsupplemented minimal agar after two replications of DNA in liquid culture. ○ = viable number; ● = µg DNA/ml; ■ = mutants/ml. on MM; ▲ = mutants/ml on Try$^+$MM

Fig. 4. The removal of BUDR from the medium after *tryD-79* cells had undergone somewhat more than one DNA replication and resuspension in fresh media devoid of the mutagen. Mutants (*try*$^-$ → *try*$^+$) still appeared on the unsupplemented minimal agar (MM). The concentrations of the analogues were 200 µg BUDR/ml. and 10 µg FUDR/ml in the presence of metabolites. ○ = viable number; ● = µg DNA/ml.; ■ = mutants/ml. on MM and ▲ = mutants/ml. on Try±MM

method of showing that mutations are induced after the removal of BU may be demonstated by using bacteriophage whose DNA contains BU. One could investigate the probability of mutation during phage DNA synthesis in bacteria free of BU. If the analogue is found to be mutagenic under these conditions, one could be sure that the mutations resulted from pairing-errors during the replication of phage DNA containing BU.

To conclude, BU-induced mutations occurred concurrently with DNA replication and, as the model of mutation by a transitional change predicts, a total of three DNA replications were necessary for the establishment of a heritable change

in genotype. Both mutant phenotypes and mutant genotypes developed at the same time. These experiments have been confirmed by STRELZOFF (1960) employing *E. coli 15t⁻* with auxotrophic markers produced with AP. She has similarly demonstrated that three DNA replications may be required before prototrophs appeared on unsupplemented minimal plates.

II. Time and mode of appearance of AP-induced mutants

In contrast to what was found with BUDR, AP-induced prototrophs appeared only after a single DNA replication in liquid. This was true of populations of

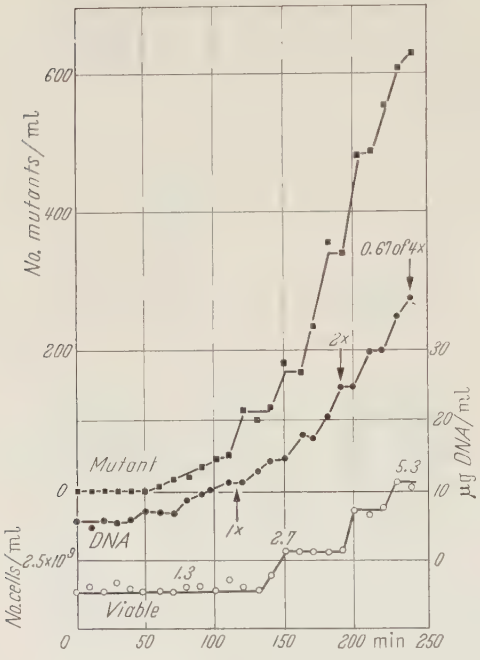


Fig. 5

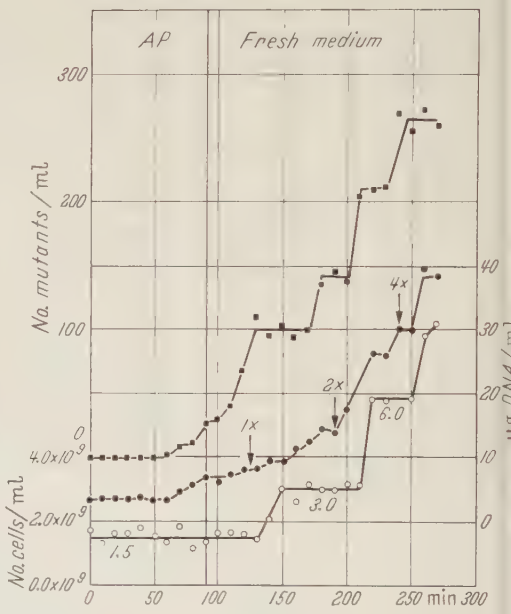


Fig. 6

Fig. 5. Time and mode of appearance of AP-induced mutations (*try⁻ → try⁺*) of *Salmonella typhimurium* strain *tryD-10*. The small cell fraction obtained by filtration was continuously exposed to 200 µg AP/ml. and followed every 10 minutes for 4 hours. The mutants as determined on unsupplemented minimal agar appeared as soon as the DNA began to replicate. ○ = viable number; ● = µg DNA/ml; ■ = mutants/ml. on MM

Fig. 6. The effect of AP when present only during the first replication cycle on the mutation of *Salmonella typhimurium try⁻ → try⁺*, tested in populations of *tryD-10* whose division had been synchronized by fractional filtration. The small cell fraction was resuspended in 200 µg AP/ml., after 90 minutes the mutagen was removed. ○ = viable number; ● = µg DNA/ml, ■ = mutants/ml. on MM

tryD-10 and *-79* bacteria whose division had been synchronized by fractional filtration. The small cell fraction was exposed to 200 µg AP/ml. and was followed every 10 minutes for 3—4 hours. The mutants as determined on unsupplemented minimal agar appeared as soon as DNA began to replicate. This was found whether AP was present continuously (Fig. 5) or only during the first replication (Fig. 6) and when the cells were allowed to replicate their DNA without cell division in the presence of inhibitory concentrations of AP (Fig. 7).

In the synchronization experiments, one notices that the appearance of the mutants as determined on unsupplemented minimal agar coincided with the mode

of DNA replication and not with the increase of the viable parents. While the total *try*⁻ population divided in a stepwise fashion 5–10 minutes after the DNA has doubled, the mutants developed in the same pattern as the increase of DNA. The various breaks in the DNA curve are also evident in the mutant curve (Fig. 5 and 6). This observation is even more evident when the cells were allowed to replicate their DNA without cell division in the presence of 500 μg AP/ml (Fig. 7). In the experiments reported here the pattern of DNA increase appeared to be somewhat discontinuous with two major steps or phases. This was observed in control as well as experimental AP-treated populations. This fashion

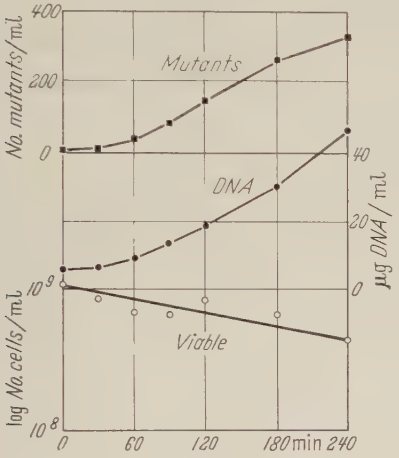


Fig. 7

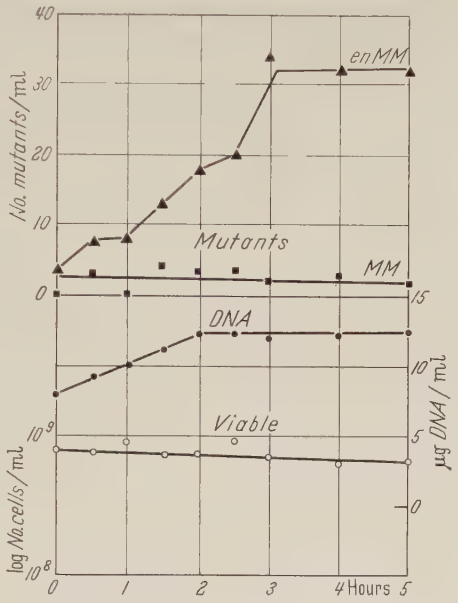


Fig. 8

Fig. 7. The effect of inhibitory concentrations of AP on the mutation of *Salmonella typhimurium* strain *tryD-10*. Under these conditions (500 μg AP/ml.) the cells were allowed to replicate their DNA without cell division. The small cell fraction obtained by filtration was resuspended in AP and followed for 4 hours. Many of the bacteria developed into long threads. ○ = viable number; ● = μg DNA/ml.; ■ = mutants/ml. on MM

Fig. 8. The effect of 200 μg AP/ml. on the mutation of *Salmonella typhimurium* strain *tryD-79* in the presence of 20 μg chloramphenicol/ml. and in the absence of tryptophan. Within 2 hours there was a net synthesis of about 50 per cent of DNA, *try*⁺ mutants appeared only on the enriched minimal plates (enMM) and not on the unsupplemented minimal agar (MM). After exposure to chloramphenicol the bacteria can no longer undergo the extra DNA replication on the unsupplemented minimal plate. It was therefore concluded that in the case of *tryD-79* the *try*⁺ phenotype is established after only two DNA replications. ○ = viable number; ● = μg DNA/ml.; ■ = mutants/ml. on MM; ▲ = mutants/ml. on enMM

of replication might very well be an artifact due for example to temperature fluctuation during filtration at room temperature and growth at 37° C, or to anoxia from concentrating the cells prior to filtration. What seems intriguing nonetheless is that AP-induced mutants appeared in a stepwise fashion just as did the increase of DNA.

Mutations from auxotrophy to prototrophy were also induced by AP when chloramphenicol was added (Fig. 8; see also Fig. 1a–b in Nakada et al. 1960). This experiment involved exposing *tryD-79* bacteria to 200 μg AP/ml and 20 μg chloramphenicol/ml. in the presence or absence of tryptophan. In 2 hours there was a net synthesis of approximately 50% DNA. Stable *try*⁺ mutants were

induced even though protein synthesis was arrested. The number of prototrophs obtained under these conditions, where DNA increased only 50%, was slightly less than half that obtained when a complete replication was allowed to occur without chloramphenicol. For *tryD-79*, AP-induced revertants appeared only on the enriched minimal plates (Fig. 8); while in the case of *tryD-10* the revertants developed even on MM (NAKADA et al. 1960).

On the basis of the chloramphenicol experiment it was assumed that for *tryD-79* the mutant phenotype is established after two DNA replications. The chloramphenicol experiment with *tryD-10*, on the other hand, showed that a 50% increase in DNA was sufficient to allow mutation and the expression of the mutant phenotype without further replication of DNA on the MM plates. The following assumptions were made for that mutant strain: (a) the try^+ character is dominant and (b) the expression of the phenotype does not require further replication. Cells which have incorporated AP in the correct site are capable of tryptophan synthesis and hence can undergo the extra divisions necessary for the establishment of the try^+ genotype. As a consequence they will give rise to try^+ and try^- cells as daughters and form a line with mutant clones rising from time to time as a result of base-pairing errors. The formation of lines can only take place as a result of the incorporation of AP or an unusual base formed only in its presence when the errors in base-pairing occur during replication. On the other hand, if a normally synthesized base is incorporated at an unusual site because of the presence of AP (Fig. 1c), or if the errors in base-pairing occur at the time of incorporation, only mutant clones are formed. This postulate was subjected to experimental test (RUDNER 1960).

The mutant strain *tryD-10* was grown in the presence of AP, following growth the cells were spread on minimal agar and were observed microscopically. Many of the cells (*ca.* 70—80%) began to divide very slowly with a generation time of $5^1/_2$ hours, eventually forming elongated microcolonies. When the increase in cell number was plotted against time, it was found that the cells increased at best arithmetically and certainly not exponentially. This process, although occurring much later and with a generation time of 23 hours, also took place on the control plates. In the case of BUDR, the mutant phenotype and genotype arise at the same time and after a total of three DNA replications (Fig. 1a). Hence no try^+ lines should be formed; indeed none were observed microscopically with *tryD-79* bacteria. Two disturbing aspects of these microscopic observations are apparent. First, too many cells began to divide to account for the 1% incorporation of AP observed (RUDNER 1961). This incorporated amount is by far too small to allow 70–80% of the cells to form lines; on the other hand, more AP molecules may have become incorporated only to be transformed *in situ* into guanine or adenine upon subsequent DNA replications (WACKER *et al.* 1960); or the AP may have been selectively incorporated at the specific mutant site. A more plausible explanation, perhaps, is that AP accelerates a natural process of residual cell division which takes place normally on minimal plates. This phenomenon may be specific for *tryD-10*. Minimal plates on which *tryD-79* bacteria were seeded after treatment with AP were also observed microscopically. No extra divisions took place on experimental or control plates. The only apparent difference

between treated and untreated bacteria was the presence of slightly elongated cells on the experimental plates (Rudner 1961).

To conclude, AP-induced mutation occurred in the presence of inhibitory concentration of the mutagen or when protein synthesis was blocked by chloramphenicol, so long as DNA synthesis was proceding even under conditions where cell division was arrested. Stable *try*$^+$ mutants began to appear at the time of commencement of DNA synthesis either when the mutagen was present continuously or for only one replication of DNA. If the residual synthesis of DNA which occurs on the MM plates is taken into account, then the mutant phenotype was established after two DNA replications in the case of *tryD-79* and after the first DNA replication in the case of *tryD-10*. Structurally AP resembles both adenine and guanine and can pair in its normal tautomeric form with both pyrimidines. It is not surprising therefore that AP:C or AP:T pairs can confer the mutant phenotype.

III. Analysis of mutant increases as a means of establishing the preferred mutagenic direction of transition inducers

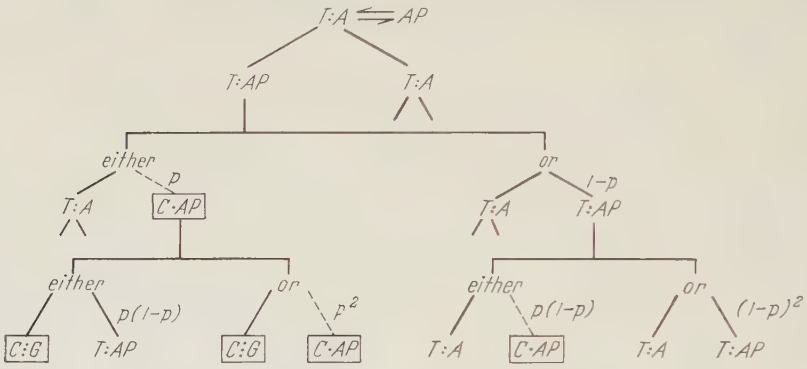
As was stated earlier, base pair transitions induced by analogues can occur by two mechanisms: 1. an *error in incorporation*, and 2. an *error in replication*. In addition, transitional changes may occur due to the incorporation of a normally synthesized base at an unusual site as a result of the effect of the analogue on the DNA precursor pool (Fig. 1). The models differ as to the times at which the enoneous base pairing takes place. It occurs during the first DNA replication cycle for errors in incorporation and at the earliest during the second DNA replication cycle for errors in replication. As a consequence the times required for the development of mutant genotype and phenotype would be different in these cases. For example, mutant clones are found at the third replication with errors in incorporation and at the earliest then with errors in replication, while a pure clone of mutants develops two replications after incorporation of a normal base at an unusual site. Mutant phenotypes can be established a generation earlier (after two replications) if the analogue is very similar to the base it resembles *e.g.*, BU:A $\cong$ T:A or AP:T $\cong$ A:T).

The fundamental difference between the two models is, however, the following one. Errors in incorporation (either of a base analogue or a normally synthesized base at an unusual site) should give rise to affected clones with $^1/_4$ mutants if no intracolonal selection is operating. Errors in replication, on the other hand, should give rise to clones which have at most $^1/_8$ mutant components if the erroneous pairing takes place at the first replication after incorporation. Usually this does not happen, and the number of mutants in such a clone is far fewer. Mistakes in incorporation are made only while the base analogue is present. Upon removal after the first replication, the mutant subclone will exponentially increase upon subsequent DNA replication. Errors in replication would, however, continue to occur throughout later replications, causing a greater than exponential increase. Since the frequency of base analogue-induced prototrophs in *Salmonella typhimurium* is not of; the order of 1–10%, a study of mosaic patterns in colonies of mutants is impossible. Instead it is possible to discriminate between

the two mechanisms on the basis of the pattern of increase of the mutants in a large bacterial population, since replication errors will continuously add new mutants to the culture while the numbers of mutants following incorporation errors will increase clonally. If these factors are taken into account, one should be able to calculate mutant increase patterns for all three possibilities. The mutagenic direction of a given transitional change could thereby be determined.

Fig. 9 illustrates the manner by which one can calculate a pattern of mutant increase for errors in replication when AP is present during one DNA replication. It can be seen that the increase pattern is neither linear nor exponential. This pattern differs significantly from the increases expected if the error were made

Errors at replication when AP is present for one replication cycle



AP Present for one cycle		AP Present continuously	
Replication in liquid	No. Mutants on MM (where 1 DNA replication occurs)	No. Mutants on MM (where 1 DNA replication occurs)	Total

a) Mutant increase pattern for errors in replication

1	n	n	n
2	2n	2n 2n	4n
3	3n	3n 4n 4n	11n
4	5n	5n 6n 8n 8n	27n

b) Mutant increase pattern for errors in incorporation

1	n	n	n
2	n	n 2n	3n
3	2n	2n 2n 4n	8n
4	4n	4n 4n 4n 8n	20n

Fig. 9. An illustration of the mutant increase pattern obtained when AP is present for one replication cycle and the error is made in replication. The chance of making a C·AP pair at the mutant site is p and the chance of T:AP is $1-p$. The amount of AP incorporated is maximally 1 % or 10^{-2} of total DNA (based on the chemical findings, RUDNER 1961). The frequency of *try*$^+$ induced by AP in these strains *(tryD-10, -79)* is about 10^{-6}. Hence if the mutation in the *try*$^-$ condition originally consisted of a T:A base pair the frequency of C·AP pairs after one replication in AP would be *ca.* $10^{-4}(p)$. While the chance of T:AP is about $1\,[(1-p) = 1-10^{-4} \cong 1]$. Furthermore, $p(1-p) \cong p$ and $(1-p)^2 \cong 1$. The chance of two successive erroneous pairings (C·AP) is p^2 (*ca.* 10^{-8}); it is therefore unlikely to occur and is not considered. The enclosed base pairs indicate those yielding *try*$^+$ phenotypes as judged by the experiments previously described. In calculating the mutant increase patterns the additional DNA replication which occurs on the minimal plate is taken into account. For these mutant strains the same increase pattern as for errors in incorporation is obtained when the mistake is due to the incorporation of a normal base at an unusual site

in incorporation of a base analogue or a normal base at an unusual site; such a pattern is essentially exponential after the initial lag of two generations. The calculations were based on a few reasonable assumptions derived from the chemical and genetic data. The amount of 2-aminopurine found is about 1% of total DNA, hence the maximal frequency of T:AP pairs at the mutant site is 10^{-2}. The frequency of AP-induced prototrophs is of the order of 10^{-6}. The change of AP pairing erroneously with cytosine is therefore about $10^{-4}(p)$. For every erroneous pairing of C with AP the chance of this pair becoming T:AP is about one $[(1-p) = 1-10^{-4} \cong 1]$. On the other hand, if the errors are made in incorporation, it becomes extremely unlikely that two successive mistakes can take place $(p^2 = 10^{-8})$, and they are therefore not considered. It was concluded that in the case of errors in incorporation additional mutants could not occur throughout later generations with anything like the frequency with which they occur when the mistake is made in replication. The increase pattern for AP takes into account the following facts:

1. Cells of *Salmonella typhimurium* when grown in defined medium contain mostly one or two nuclei (Schaechter, Maaloe and Kjeldgaard 1958). The small cell population collected by filtration is uninucleate. This nuclear number doubles upon DNA replication during which the base analogue is incorporated. As a result, a cell comes to possess the two sister double helices only one of which has the incorporated base analogue. In order for a clone of homokaryotic dominant mutants to begin to increase, the altered nucleus must segregate from its nonmutant sister. This results in a segregation delay of one division (Ryan and Wainwright 1954).

2. The dominant mutant phenotype develops after two DNA replications and since one occurs in the plate, the mutant appears at the end of the first replication in liquid. In the presence of BUDR, the same pattern of increase occurs after a lag of two generations. As a consequence, when the analogue is present for only one replication, errors in incorporation lead to an increase of *0, n, n, 2n*; an error in replication to *0, n, 2n, 3n*. The same events are considered when the mutagen is present throughout the experiment as when it is present for only one DNA replication cycle (Fig. 9).

Fig. 10 illustrates the pattern of increase of *try*$^+$ mutants in synchronized populations of *tryC-48* and *tryD-73* exposed to AP and BUDR respectively. Upon the removal of the base analogue from the culture after the first DNA replication cycle, *tryC-48* generated *try*$^+$ mutants which increase in a pattern indicative of an error in replication *(n, 2n, 3n)*, suggesting that the mutagenic direction of that transition is A:T→G:C. On the other hand, *tryD-73* is apparently reverted by the opposite transition G:C→AT:T because the mutants increased in a pattern predicted for errors in incorporation *(0, n, n)*. The difference between the two mechanisms is immediately evident from the mutant increase curve (Fig. 10). A plateau *(n, n)* is always established in the cases of an incorporation error, while the mutant curve is essentially continuous for replication errors.

The Table summarizes the direction of base-pair transitions involved in the reversion of six tryptophan-requiring mutants of *Salmonella typhimurium* induced by BU and AP as suggested from the pattern of increase of the *try*$^+$

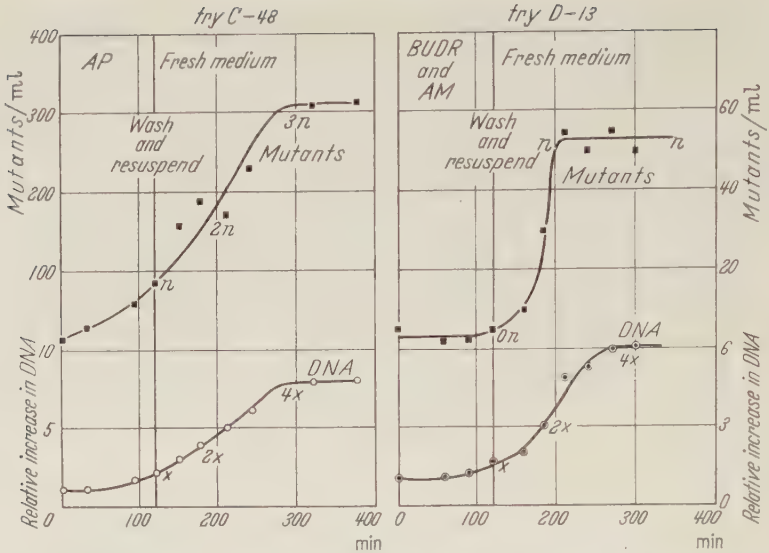


Fig. 10. The pattern of increase of try^+ mutants in synchronized populations of try^- *Salmonella typhimurium* exposed to AP or BUDR and aminopterin (AN) for one DNA replication cycle. After the removal of the base analogues and resuspension in fresh medium, *tryC-48* generated try^+ mutants which increased in a pattern *(n, 2n, 3n)* calculated for an error in replication. On the other hand, *tryD-73* generated try^+ mutants which increased in a pattern *(o, n, n)* indicative of an error in incorporation. ○ = μg DNA/ml.; ■ = mutants/ml. on MM

Table. *A summary of the direction of base-pair transitions involved in the reversion of tryC and -D mutants of Salmonella typhimurium induced by base analogue incorporation as suggested by the pattern of increase of try^+ mutants in synchronized populations*

Mutant	Origin of try^- strain	Mutagen, $try^- \rightarrow try^+$	Pattern of mutant increase	P by χ^2 test	Nature of error	Direction of transition
C-3	ultraviolett	AP	n 2n 3n	0.16	replication	A/T → G/C
C-48	spontaneous	BUDR	n 2n 3n	0.90	replication	A/T → G/C
		AP	n 2n 3n	0.58		
			n 4n —*	0.40		
D-7	ultraviolett	BUDR	0 n n	0.74	incorporation	G/C → A/T
D-10	pontaneous	AP	n 2n 3n	0.93	replication	A/T → G/C
				0.89		
			n 4n 11n*	0.89		
				0.15		
D-73	AP	BUDR	0 n n	0.65	incorporation	G/C → A/T
		AP	n n 2n	0.68		
			n 3n —*	0.65		
D-79	AP	BUDR	0 n 3n*	0.89	incorporation	G/C → A/T
		AP	n n —	0.98		
				0.70		

* The mutagen was present continuously instead of for only one DNA replication.

mutants. The numbers of mutant colonies scored in experiments employing synchronized populations were subjected to χ^2 test where the expected n was

calculated from the cumulative numbers observed after three replication cycles in liquid divided by the number of n's in the theoretical series. In all six cases the numbers of observed mutants were not significantly different from the numbers predicted on the models of errors in replication or incorporation. Furthermore, in the cases where both mutagens were applied to the same mutant strains *(C-48, D-73* and *D-79)* the same mutant increase patterns were obtained. This suggests that AP makes incorporation errors in the same *try*$^-$ strains as does BUDR which usually replaces thymine; therefore, AP must habitually replace adenine. On the basis of the chemical data obtained it was possible to conclude that AP can become incorporated into the DNA of *Salmonella* (Rudner 1961), but it was impossible to conclude which of the purines it normally replaces. These mutagenic analyses clearly demonstrate that AP is an adenine analogue and only rarely can it pair erroneously with guanine.

These results further suggest that in the *try-C* and *-D* loci of *Salmonella typhimurium* those mutant sites which respond to base analogues must have arisen as point mutations. Out of the six strains tested, 4 were of spontaneous or U.V. origin, suggesting that base transitions can occur spontaneously and also under the mutagenic influence of U.V. light. Strelzoff (1960) also observed the two different patterns of mutant increase for *E. coli 15t*$^-$ with auxotrophic markers produced with AP. Another interesting conclusion can be drawn from these analyses. As was stated earlier, the *try*$^+$ mutants so far studied in *Salmonella* appear on MM plates one replication after the incorporation of AP and two replications after the incorporation of BUDR. Therefore, *try*$^+$ phenotypes are presumed to be given by C:AP but not by BU:G. Two exceptions to this observation were noticed. *TryC-48*, which yields mutants in response to BUDR after only one replication, shows that the *try*$^+$ phenotype may sometimes be given by BU:G. This implies that the information for synthesizing the enzyme tryptophan synthease may be in one strand: the one containing G at the relevant site in the case of *tryC-48* and the one containing C in the other mutants which reverted by the transition A:T→G:C. The second case was the behavior of *tryD-10* in the presence of AP which was discussed in Section II. In the absence of knowledge of the influence of neighboring pairs, it is not possible to decide whether the genetic information resides in one or in two DNA strands. Nor do these analyses provide information as to wheter the base pairs are in an inverted $\overset{\text{T}}{\underset{\text{A}}{\vdots}}$ or upright $\overset{\text{A}}{\underset{\text{T}}{\vdots}}$ position.

D. Discussion

The mutation model by transitional change is based entirely on the Watson and Crick (1953) molecular structure for DNA. To date, no evidence except for that in one report has been incompatible with its structure and replication properties. Cavalieri's (1960) experiments are the only ones which imply that DNA replication in vivo does not proceed by a separation of the two strands and the complementary formation of new strands upon these. Rather, he supposes that in some manner old DNA double helices are wholly conserved, while progeny DNA double helices are completely new. His conclusion is based solely upon the kinetics of DNase digestion of DNA units with a molecular weight of 1.3×10^6 which were obtained after heating in $CaCl_2$. These were presumably still double

units. Some caution about his conclusions must be expressed, especially when a vast literature of biochemical and genetic data reflect the two-stranded nature of bacterial and phage DNA and its semi-conservative mode of replication.

Mutation by a transitional change includes replication of DNA as an essential step. A mutation from this point of view is that rare event in which at a particular site in a replicate chain, a purine or pyrimidine base non-complementary to the nucleotide in the parental chain happens to be incorporated. The first double stranded DNA molecule resulting from this mutational change should thus contain one nonmutated parental strand and one mutated strand; in other words it should be "heterozygous" for the mutant site. Upon subsequent DNA replication of this heterozygous molecule, the two complementary chains would separate from each other to generate two "homozygous" daughter molecules, one mutant and the other non-mutant. It is interesting to note that such mutational heterozygotes have been observed in bacteriophage T2 and after treatment with BUDR and nitrous acid (PRATT and STENT 1959, VIELMETTER and WIEDEN 1959, and TESSMAN 1959). These mutational heterozygotes are expressed as mottled *r*-plaques (mixed *r* and r^+ clones). The interpretation that this heterozygosity reflects the two-stranded nature of phage DNA is further strengthened by TESSMAN's (1959) findings that no such mutational heterozygotes were observed after the induction of mutations with nitrous acid in phage $\varnothing$ X *174*, a virus whose DNA is known to consist of only a single polynucleotide strand (SINSHEIMER 1959).

Further evidence that replication of DNA is an essential step in the process of mutation induced by base analogues is presented in this paper and is based on studies on tryptophan-requiring mutants of *Salmonella typhimurium*. This evidence takes several forms: (a) more mutants were obtained when treated cells were plated on supplemented minimal plates, (b) when cells are not allowed to divide in the presence of inhibitory concentrations of AP or BUDR, mutants were still induced so long as DNA synthesis took place, and (c) mutations can be induced with AP while DNA synthesis is proceeding even though protein synthesis is blocked by chloramphenicol. In the latter case, RNA also increased almost threefold (NAKADA et al. 1960). The RNA synthesized in the presence of chloramphenicol is considered biologically inactive and could not account for the induced mutations since it is known to degrade (NEIDHARDT and GROSS 1957: HOROWITZ et al. 1958). Inhibition of RNA synthesis with an antimetabolite such as 5-OH uridine or the starvation of a uracil-requiring mutant for that growth factor under the same experimental condition will probably provide a better experimental demonstration. Other investigators have shown that mutations can be induced in bacteriophage *T2* by exposure of infected cells to BU while DNA synthesis is proceeding with the inhibition of protein synthesis by chloramphenicol (BRENNER and SMITH 1959, LITMAN and PARDEE 1959). The findings that DNA synthesis is essential for base analogues mutagenesis further coincide with LITMAN and PARDEE's (1960b) kinetic studies of BUDR induced mutagenesis in bacteriophage *T2*. They reported that BUDR became effective as a mutagen at the time of commencement of phage DNA synthesis (10 minutes after infection); furthermore, the mutagenic action of BUDR could be exerted at any time thereafter as long as phage DNA continued to be synthesized.

It is extremely difficult to comment on the mode of DNA replication in these synchronized populations since the experiments were not designed to answer this question. DNA was determined colorimetrically employing the Dische reagent and the pattern of its increase appeared somewhat discontinuous with two major steps. This method is less sensitive than exposure to tritiated thymidine for short periods (Schaechter et al. 1959, Abbo and Pardee 1960). Maruyama and Yanagita (1956b), who devised this method of synchronizing bacterial populations by fractional filtration, also reported a non-continuous or stepwise synthesis of DNA, RNA and protein throughout the division cycle. Since then the same has been observed in other bacterial populations (*Salmonella typhimurium* and *Alcaligenes faecalis*) by Maruyama and Lark (1959) and Lark (1960), who further demonstrated that the periodicity of DNA and RNA synthesis is destroyed by the addition of deoxynucleotides. The authors stress that DNA synthesis occurs only during certain "permitted" portions of the division cycle. It therefore seems that the timing of DNA synthesis in bacteria is still a controversial question, and the critical experiment is yet to be performed.

In order for a base analogue to induce a transitional change it has to become incorporated into DNA where upon it may act as a tautomeric mutagen. Chemically it has been demonstrated that BU quantitatively replaces thymine, while we may conclude that AP replaces adenine. It was stressed throughout this report that permanent mutations by a transitional change are not caused merely by the replacement of a base in DNA by its analogue. Mutations are rather induced by errors in base pairings in which the analogue may engage in more or less frequently. The fact that only 10% mutants are obtained when almost all the thymine residues in bacteriophage *T2* DNA are replaced by BU (Liltman and Pardee 1960a) supports this conclusion. Errors in pairing can come about when the base analogue undergoes tautomeric shifts. To date there is really no physico-chemical evidence that BU and AP are more stable in their rare enol or imino forms, hence bringing about the replacement of an adenine-thymine pair by a guanine-cytosine pair or *vice versa*. The best indirect evidence that this is so comes from the fact that BU and AP are highly effective in producing reversions of the mutation they had induced originally. Curiously enough, BU is particularly effective in reverting AP induced mutations and *vice versa* (Freese 1959a). It appears as if each base analogue produces changes in one direction more often than in the other. What seems important to establish is whether a given base analogue has a preferred mutagenic direction or whether it can induce transitions in both directions (A:T $\rightleftharpoons$ G:C) via errors in replication or in incorporation. Freese (1961) strongly believes that a preferred mutagenic direction does exist; for example, in the case of BU, mistakes occur in incorporation (G:C $\rightarrow$ A:T) more frequently than in replication, according to him. This, he believes, is due to the electronegativity of the bromine group which may induce tautomeric shifts in BU. Once the BU has become a member of a DNA strand the negative charge of its bromine may become partially reduced by neighbouring groups and no further shifts can occur. Although reasonable, this explantation is still very speculative. It seems equally as plausible that the chance of BU pairing erroneously with cytosine is the same whether it occurs in the initial DNA replication cycle or in subsequent ones. This is supported by the findings that two distinct

patterns of reversion increases were observed in the tryptophan-requiring strains of *Salmonella typhimurium* induced by BUDR.

To determine the nucleotide pair(s) which constitute a mutational site, one has to establish the direction of a transitional change. This can be done either by distinguishing between the two types of errors, as was attempted here, or by applying agents which have a specific and known chemical action on DNA bases, and are capable of reverting base analogue transitions. The latter approach was adopted by FREESE (1961). The chemicals used were: 1. the alkylating agent, ethyl ethanol sulfonate (EES) which apparently ethylates only the 7-position of guanine, 2. hydroxylamine (HA) which reacts specifically with cytosine and uracil but not with thymine and purines, and 3. low pH or high temperatures which depurinates guanine more readily than adenine. According to Freese these agents specifically produce transitions from a G:C pair to an A:T pair but not *vice versa*. EES, for example, was highly effective in reverting most of the AP-induced *rII* mutants, while it had no effect on most of the BU-induced mutants. His conclusion was, therefore, that most AP mutants had a G:C pair since they could be induced to mutate by EES while the BU mutants had an A:T pair and were inert to the treatment with the alkylating agent. He further concluded that BU acted by errors in incorporation (as was found for *D-79*, *D-73* and *D-7*). How universal this conclusion is remains to be seen.

So far it has been assumed that transitional changes of base pairs can occur anywhere along the DNA molecule under the mutagenic action of base analogues and nitrous acid. Genetic and chemical observations indicate that this is not the case. The probabilities of base transitions are not random. They seem to depend on the kind of base pair and also its position within the DNA molecule, as if neighbouring nucleotide pairs and their special sequences determine whether the change will occur or not. The latter is apparent from the great variation of response to the mutagens observed among the various inducible-sites in *Salmonella typhimurium* (RUDNER 1961). The existence of specific "hot spots" which arose with BU and AP again seen to indicate that the mutability of a given nucleotide pair is dependent upon its position in the genome. When the number of genetic sites at which n mutations have been observed (for independently isolated *rII* mutants) was compared to a Poisson distribution, a significant deviation was found (FREESE 1959c). This further points to the non-random effect of base analogues. Specific genes may also influence the probability of base transitions along the genome. A mutator gene in *Salmonella typhimurium*, localized on the chromosome and known to increase the frequency of mutations of many genes, is an example. It was demonstrated that the presence of such a mutator gene greatly increased the frequency of transitional changes among histidineless sites of spontaneous origin. These sites did not respond to base analogues and nitrous acid in the absence of the mutator gene. In its presence they were sensitive to the treatment as if this gene, in some way, facilitates an event leading to a transition (KIRCHNER 1960). Perhaps the presence of mutator gene weakens H-bonds or causes hydrolysis of the sugar-base bond (depurinations, depyrimidinations) so as to allow a more frequent incorporation of a normal or an unusual base at the mutant sites.

Chemical analyses of DNA from various sources further indicate that purines and pyrimidine bases are not distributed at random but rather follow a certain

pattern. SHAPIRO and CHARGAFF (1957) reported that at least 70% of the pyrimidine in various DNA samples, including some from *E. coli* occur as oligonucleotide "tracts" containing three or more pyrimidines in a row and similarly there are "tracts" of purines. These "tracts" are flanked by "solitary" purine and pyrimidine bases. Recently, the same authors (SHAPIRO and CHARGAFF 1960) reported that approximately one-third of the incorporated BU in the DNA or *E. coli t⁻ (I)* has been found segregated between purines in the form of solitary units. Although the report contains some disturbing facts, especially with respect to the general distortion of the nucleotide sequence which was observed, it does point out that an exclusion principle plays a role, so that certain nucleotide neighbours are tolerated by BU and others are not, or rarely are tolerated. If this is so and if it is assumed that a single DNA strand is adequate to convey genetic information, one can visualize that a mutational site which responds to BU is probably a solitary pyrimidine flanked on both sides with tracts of purines. The neighbouring purines may have different effects on the mutability of that particular site. Such effects may be produced by the differences in H-bond strength of adjacent pairs, that of A:T being less than that of G:C (FREESE 1961). Ideally, one would like to determine directly the nucleotide pairs which constitute a site by comparing chemically the nucleotide sequence of wild type and mutant DNA. This is impossible at least at the present time. Instead, one must proceed more indirectly and derive a molecular picture by comparing genetic and chemical observations as was attempted in this report.

Summary

The model of mutation by transitional change (FREESE 1959) predicts that a heritable change in genotype is established when two replications of DNA succeed the initial incorporation of an analogue. The model was tested in populations of *Salmonella typhimurium* strains *tryD-10* and *tryD-79* whose division had been synchronized by fractional filtration. Mutation from auxotrophy to prototrophy *($try^- \rightarrow try^+$)* induced by 5-bromodeoxyuridine (BUDR) and 2-aminopurine (AP) occurred in accordance with DNA replication. Two subsequent DNA replications were necessary to obtain BUDR-induced prototrophs in *D-79*, one subsequent DNA replication was required for AP-induced prototrophs in *D-79*, while no subsequent DNA replication was necessary for AP-induced prototrophs in *D-10*. This was observed whether the mutagens were present continuously or during only the first replication and also when the cells were allowed to replicate their DNA without cell division in the presence of inhibitory concentrations of the base analogue or when protein synthesis was blocked in the presence of chloramphenicol. A statistical analysis of the patterns of mutant increase observed for six mutant strains was used to distinguish between "errors in replication" and "errors in incorporation" induced by the base analogues and thereby the base pair at the mutant site was identified.

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COMPOUND LOCI AND COINCIDENT MUTATION IN *NEUROSPORA*

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With 2 Figures in the Text

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In many instances where back-mutation of an auxotrophic mutant has been studied in detail, revertants of intermediate phenotype have been detected (e.g., Giles, 1953, 1956, 1958; Fincham, 1957). Many of those revertants which seemed identical with the wild type as judged by one criterion, for example, growth on minimal medium, differed when examined by other criteria, such as enzyme activity or accumulation of particular metabolites (Giles, 1956, 1958). Usually, this deviation from the normal phenotype was in the direction of the parental mutant one. The several classes of partial or intermediate type reversions segregated as if linked to the mutant locus. Moreover, Ishikawa (1961) has demonstrated recently the back-mutability of two *ad-8* mutants of *Neurospora* which probably involve deletions.

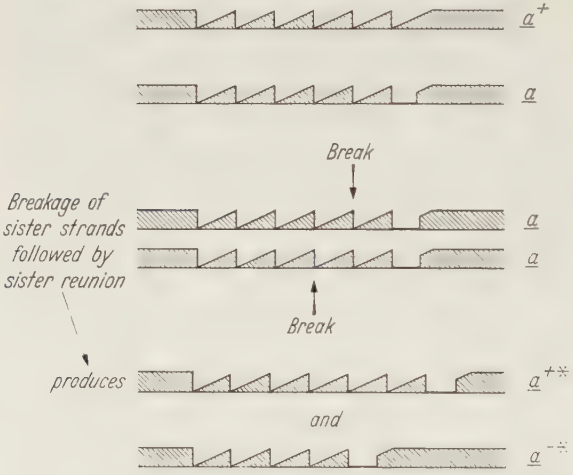

Fig. 1. Representation of back-mutation by unequal sister-strand exchange

These observations suggest that often back-mutation may be a more complex phenomenon than a simple base-substitution of, say a guanine for an adenine or of a thymine for a cytosine deoxyribonucleotide (Freese, 1961; Rudner, 1961) and that back-mutation to the normal or wild type condition may not be a sufficient criterion for classifying a heritable change as a point mutation.

Mechanism of back-mutation. Sister strand exchange hypothesis. If revertible loci consist of multiple repeat segments and a mutation from wild type to mutant consists of an alteration, e.g., a deletion, in one or more of the segments, back-mutation could be explained by a sister strand exchange process. While such sister strand exchanges have not yet been demonstrated in *Neurospora*, they have been found to occur with a frequency of the order of one per chromosome per cell division in those plant species studied (Taylor, 1958). A proportion of the exchanges might be expected to be unequal, leading to the production of one duplication and one deficiency strand (Fig. 1). We can imagine, however,

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that the process need not be reciprocal. Sister reunion of one pair of strands need not be dependent on the reunion of the other pair. If the duplication strand resulted in the production of an effective enzyme and consequently a wild type-like phenotype, such a strand would be selected for.

A hypothesis of back-mutation such as this leads to certain predictions and explains certain observations:

1. It predicts that reversion usually would not be a precise process. This could explain the range of partial and intermediate type reversions observed.

2. But just as small duplications insufficient in size to replace completely the segment lost by mutation from, say a^+ to a^-, would be expected to produce a partial reversion phenotype, so larger duplications which resulted in an excess of repeat segments might also have some effect on the phenotype. Moreover, such a phenotypic alteration might not be related to the physiological activities of the normal a^+ gene.

3. According to the "**central dogma**", if the wild type gene consists of a number of repeat segments, i.e., a number of identical base sequences arranged in a linear order, then the protein/enzyme whose structure is determined by such a gene might also be expected to have some repetition in its structure.

4. If the exchange event leading to the formation of an a^+-like phenotype were of the reciprocal sister-strand type, the complementary genome should be detectable, given suitable technical procedures.

With these ideas in mind, an attempt was made to recover the hypothetical complementary strands resulting from mutation in single haploid nuclei (prediction 4, above) as heterocaryons. Because of the additional possibility that new auxotrophic characters associated with the back-mutational event might arise (prediction 1, above), it was desirable that a selective procedure be used which, while selecting back mutant cells, did not exclude newly arisen novel auxotrophic cells. These experimental requirements were met by using a histidine strain which does not grow on complete media (Mathieson and Catcheside, 1955) and selecting for revertants on a rich complete medium as well as on minimal medium.

Materials and Methods

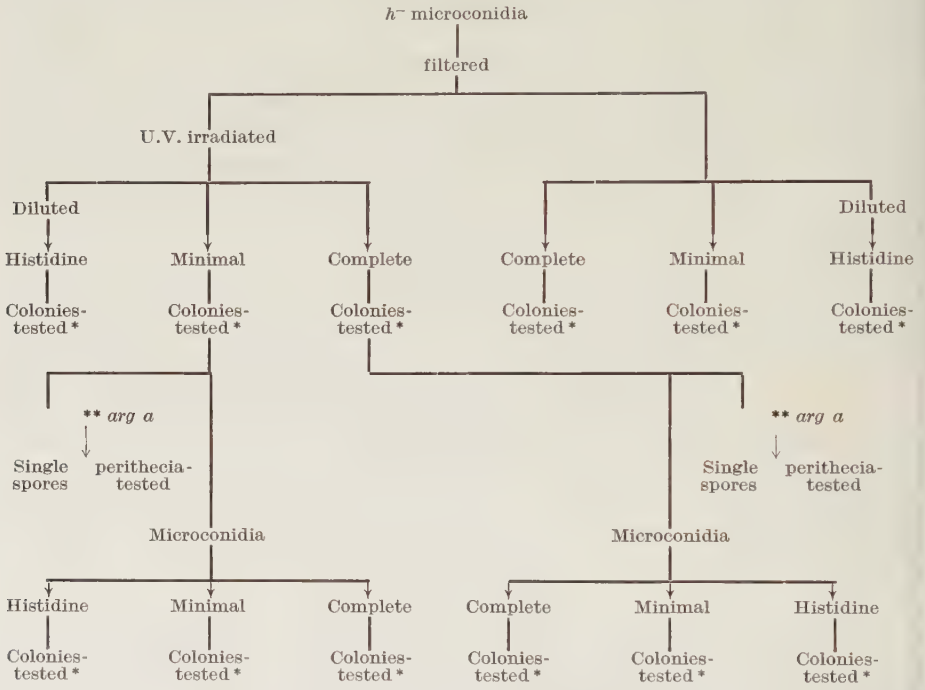
Materials. The histidineless strain *Gg59* allelic with the *his-3* strain *K26* (Mathieson and Catcheside, 1955), was used in the experiments. *Gg59* was isolated following ultra-violet irradiation of conidia from the **cmsa** strain (Grigg, 1958) and had the genotype *al-2, col-1, m su-m, acon-t* (Barratt et al., 1954; Grigg, 1958, 1960a, b). In crosses with *K26*, *Gg59* segregated no prototrophic recombinants in 11,000 progeny scored. Nor did it form a heterocaryon with this strain. Like *K26* it accumulated histidinol in the medium when grown on limiting histidine. The **cmsa** strains can be induced to produce microconidia almost exclusively in high yield and viability by growing the fresh inoculum at 35° for two days, followed by further growth at 25°. Microconidiation commences about 5—7 days after inoculation.

Irradiation Experiment. A clonal culture of the *Gg59 his* stock was established from a single microconidium. This culture was induced to produce microconidia. The uninucleate spores were scraped off tube slopes into distilled water and filtered through two grades of sintered glass filters of known pore size (Jobling, No. 1 and 3) to remove in turn pieces of myceleium and other debris and the few contaminating macroconidia. The microconidia were then washed twice in distilled water and divided into two portions. One was irradiated with ultra-violet and plated in low concentration into minimal and complete medium (approx. 3,000 viable conidia per ml of medium). The other portion was plated in similar

media as control and like the treated group, incubated at 25°. Viability determinations were made of control and irradiated conidial suspensions on histidine medium (100 μg/ml of histidine). Fifty single conidial cultures were isolated from the viability plates and tested later for heterogeneity of phenotype as measured by their growth characteristics.

Reversions were counted after 3, 4 and 5 days of incubation, immediately subcultured into tubes of complete medium and tested on minimal, complete and histidine media at 25° and 35°.

Testing for heterocaryosis. Microconidia from each of the reversions were washed twice and plated on minimal, complete and histidine media at a dilution sufficient to give 20 to 50 colonies per plate. Fifteen of these presumably homocaryotic colonies were picked off each plate into tube slopes and their growth behavior examined on minimal, complete and histidine media at 25° and 35°. To ensure that they were in fact homocaryotic, further samples of microconidia were taken from the colonies, washed, diluted and plated on minimal, complete and histidine media and the resulting colonies tested.



* Tested on Minimal, Complete and Histidine at 25° and 35°.

** Microconidia from selected colonies plated on histidine and complete to check that they are homocaryotic. Homocaryons x *arg a* and progeny tested.

Fig. 2. Diagram showing design of experiment

Segregation of reversion phenotype. At the time that the reversions were subcultured, prior to testing their growth behavior, they were crossed to an *al-2*$^+$, *arg-3*, *a*, strain. Twenty ripe perithecia from each cross were isolated, washed free of adhering ascospores and squeezed to eject the contents and 30 to 40 ascospores taken at random and tested for the segregation of the mutant character, mating type, *arg-3*, *al-2*, and *col-1*. Since normally the perithecia contain the meiotic products of the fusion of only two nuclei, such a perithecial analysis should differentiate a back-mutation from an unlinked suppressor type reversion. In the latter case, one-quarter of the spores should be histidine requiring. As well, it offered an independent check on whether or not the original reversion was a heterocaryon. The design of the experiment is set out in Fig. 2.

Media. (a) Minimal medium. Westergaard's medium (Westergaard and Mitchell, 1947) was used throughout, at p_H 5.6 for plating or testing cultures, or at p_H 6.5 for crossing. "Histidine medium" was minimal supplemented with 100 μg/ml of L-histidine.

(b) Complete medium. Horowitz's (1947) complete medium.

Results

The dose of ultra-violet irradiation used killed 90% of the erstwise viable conidia and significantly increased the yield of reversions (Table 1). Note that the viability of the unirradiated conidial sample was 10% of the total (haemocytometer) count.

Of the first fifteen reversions examined, ten were clearly heterocaryotic and the other five, whose status is in some doubt, probably were heterocaryotic also. Several of these latter cultures had an unstable "partial revertant" component.

The phenotypes of the ten stable heterocaryons are presented in Table 2. With the exception of *B2-M*, all had nuclear ratios ranging from 0.2 to 0.7, but which did not differ significantly from 0.5. They resembled the parental h^+ **emsa** strain in phenotype, except for the inhibition of growth on complete medium at 35°.

Reversions isolated on minimal medium

Each of the four heterocaryotic reversions from the minimal plates consisted of a histidine component and a histidine independent one.

h^+ components. The histidine independent components ranged in growth behavior from close to that of the **emsa** h^+ stock, e.g., *A1-M*, to that of *A5-M* where the h^+ component *A5-M2* was completely inhibited on complete medium at 25° and 35°, to the unusual h^+ component *A4-M2* of *A4-M* which grew only on complete medium and not on either minimal or histidine media. That it was h^+ was indicated by the h^+ phenotype of the heterocaryon (*A4-M1*) it formed with the h^- component. Further investigation indicated that the complete requirement could be satisfied by pantothenate. The other h^+ components, though able to grow well on minimal at 25° and 35° and on complete medium at 25°, were unable to grow on complete at 35°.

h^- components. The h^- components *A1-M1* and *A7-M1* isolated from two of the heterocaryons grew less well on histidine than the parental *Gg59* strain. The other h^- components seemed relatively normal. All the h^- components like the parental *his* strain accumulated histidinol in the mycelium and medium when grown on medium containing a limiting amount of histidine.

The status of isolate *B2-M* which had an *h^+-like* phenotype is in some doubt. Probably it is a heterocaryon since all forty-five homocaryotic colonies resulting from the plating of *B2-M* microconidia on minimal histidine or complete media had a very different phenotype (see *B2-M1* in Table 2) from that of the *B2-M* strain. As well, there was an excess of colonies on the minimal plates ($\bar{x} = 100 \pm 7$) compared to the complete ($\bar{x} = 19 \pm 1$) and histidine ($\bar{x} = 41 \pm 3$) plates. The homocaryons (*B2-M1*) grew poorly on minimal and not at all on complete or histidine media. No component with a complementary phenotype was detected. If *B2-M* was a heterocaryon, either the nuclear ratio must deviate considerably from that of the other heterocaryons ($p = 0.5$) or perhaps one component may behave as an indispensable function mutant (Atwood and Mukai, 1953) and not grow on any of the media used (cf. component 1 of heterocaryon *A6-C*).

Table 1. *Mutation experiment with a h strain using minimal and complete as selective media*

	Control		U.V. irradiated	
	Minimal	Complete	Minimal	Complete
No. h^+ colonies	0	4	8	8
No. $h^+/10^6$ viable cells	0	1	26.7	26.7
Survival	1.0	1.0	0.1	0.1

Table 2. *Growth of heterocaryons and their component strains at 25° and 35° on three different media*

Strain	25°			35°			Micro-conidial ratio
	Minimal	Complete	Histidine	Minimal	Complete	Histidine	
A1-M	++++	++++	+++	++++	−	++	
Components 1.	−	−	+	−	−	+++	3
2.	++++	+++	++	+++	++	++	7
A4-M	++++	++++	+++	+++	−	+	
Components 1.	−	−	+++	−	−	++	12
2.	−	++++	−	−	++++	−	14
A5-M	+++	+++	+++	++	−	++	
Components 1.	−	−	+++	−	−	+++	3
2.	++++	+	+++	++	−	+++	4
A7-M	++++	+++	++++	++++	−	++	
Components 1.	−	−	++	−	−	++	8
2.	++++	+++	++	++	−	++	8
B1-M	++++	++	++	++++	−	+	
Components 1.	−	−	+++	−	−	++	2
2.	++++	+++	+++	+++	+	+++	8
B2-M	+++	+++	+	+++	−	+++	
Components 1.	+	−	−*	+	−	−*	
2.	?	?	?	?	?	?	
A1-C	++++	+++	+++	++++	−	++++	
Components 1.	−	−	+++	−	−	+++	9
2.	−*	−*	−*	++	++	−	4
A3-C	++++	+++	+++	++			
Components 1.	−	−	+++	−	−	+++	4**
2.	−	+++	−				2
A6-C	+++	+	+++	++	−	++	
Components 1.	−*	−*	−*	−	−	−	35
2.	+++	+++	++++	+++	−*	+++	25
B5-C	++++	++	+++	+++	−	++	
Components 1.	−	−	++++	−	−	+++	6
2.	+++	+++	+++	−*	−	−*	3
cmsa h^+	++++	++++	+++	++++	++++	+++	
Gg 59 h^-	−	−	++++	−	−	++++	

* No macroscopic growth but conidia germinated.
** Perithecial ratio.

Reversions isolated on complete medium

In general, the heterocaryons which arose on the complete medium containing irradiated conidia were similar to those on the minimal plates. All grew well

on minimal medium, but were inhibited on complete medium at 35°. The components isolated from the heterocaryons had a variety of phenotypes some of which were different from those described above.

h⁺ components. One *h⁺* component (*B5-C2*) had a phenotype similar to **emsa** *h⁺* at 25° but showed a temperature sensitivity at 35° which was not altered by the addition of histidine or of the components of complete medium to the growth milieu. Another (*A1-C2*) of even more extreme phenotype showed scarcely any growth on any of the test media at either 25° or 35° and *A3-C2* grew only on complete medium. The histidine components present with each of these three novel *h⁺* nuclei in the heterocaryons were normal and resembled the parental *his* strain.

The *A6-C* heterocaryon, however, whose growth was inhibited on complete medium at both 25° and 35°, was unique in that growth of neither component strain gave a measurable response to histidine supplementation. One component (*A6-C1*) grew very poorly on minimal at 25°, but not at all at 35° and might almost be classified as an indispensable function lethal. Growth of the other (*A6-C2*) was inhibited on complete medium at 35° but otherwise resembled that of the **emsa** *h⁺* strain.

Tests of a sample of fifty *h⁻* clones derived from irradiated conidia failed to reveal any such slow growing *h⁻* strains.

Genetic segregation of the novel partial revertant characters

The **emsa** strain, while useful for plating experiments of the type performed here in that it could be induced to form either macro- or microconidia, proved rather infertile and could be used only as a male parent. Generally, germination of ascospores was poor. As well, the presence of the colonial character confused the scoring of subtle growth rate difference.

Nevertheless, segregation of many of the extreme phenotypes described could be demonstrated. With one doubtful exception, no evidence for any of the *h⁺* phenotypes being due to suppressor type mutation was obtained. The number of ascospore cultures tested, however, was not great and more data are required to eliminate the possibility of suppressor mutation being responsible.

The restricted growth characteristics of the heterocaryon components *A1-C2* and *A6-C1* segregated as a single gene linked to mating type[1] (with no recombinants in 16 and 9 progeny scored), and so did the temperature sensitive character of *B5-C2* (no recombinants in 15 progeny). Two other aberrant *h⁺* phenotypes which segregated as single genes were the unknown growth requirement of *A3-C2* and the complete medium inhibition of *A5-M2*. The pantothenic acid requirement of *A4-M2* segregated as if unlinked to *arg-3* which is close to the *his-3* locus. This suggested that the *h⁺, pan⁻* phenotype might be due to a suppressor type mutation with the *pan⁻* gene being the suppressor. However, no histidine requiring progeny were found amongst 87 progeny spores of a cross of the *A4-M2* type homocaryon with *arg-3A*, so it is unlikely that an unlinked suppressor mutation was responsible for the histidine independent, pantothenic acid phenotype.

[1] With which *h⁻* is closely linked.

Discussion

Origin of the heterocaryons

The predominance of heterocaryons amongst the prototrophic colonies indicated a bipartite genome in many or all of the cells from which the colonies derived. That these arose from single nuclei in which the chromosome was double appears more likely to us than the alternative explanations that the heterocaryons arose from mutation in

1. multinucleate macronidia, or
2. uninucleate conidia,

followed by fusion of the back-mutant h^+ mycelium with a h^- conidium. This conclusion is based on the following results: 1. The proportion of contaminating macroconidia amongst the microconidia reaped from the culture surface was not higher than 1% and was reduced considerably below this figure by filtration through graded sintered glass filters. Moreover those contaminating macroconidia which did pass through the filters would tend to have a size distribution lower than a sample of unfiltered macroconidia and consequently, a higher proportion of uninucleate individuals than a population of unfiltered macroconidia. A sample of the latter has a mean of 2.1 nuclei per conidium with about 20% having only one nucleus (Grigg, unpublished).

On the other hand the viability of microconidia is less than that of macroconidia. In this instance it was 10.0% whereas macroconidia from cultures of this age would be expected to have a viability close to 100%. As a consequence the proportion of viable macroconidia to viable microconidia would be up to ten times that of the proportion based on a total haemocytometer count. This would still be less than 10%.

2. The h^- conidia were plated in low concentration in the selective media (c. 3,000/ml) to minimize the chances of early anastomoses between germinating h^+ and h^- conidia. Since the input nuclear ratio tends to be preserved in heterocaryons (Pittenger et al., 1955) and since the nucelar ratio was, with one exception, close to 0.5, fusion between each of the h^+ conidia with h^- conidia would have to occur immediately to explain a post-mutation production of the heterocaryons. Even limited prefusion nuclear division would distort this nuclear ratio considerably.

Coincidental events

Two types of coincidental events associated with mutation of h^- to h^+ have been observed. There is the occurrence of two different mutations involving the same locus (or closely linked ones) in each component of two of the ten heterocaryons studied in detail. Thus in heterocaryon *A 1-M* a mutation of h^- to h^+ has occurred accompanied by the simultaneous mutation of h^- to h^* where h^* has growth characteristics different from h^-. And in *A 6-C* while one component had a *h+-like* phenotype the other had a semilethal phenotype on all test media.

The second type of coincidental mutational event was observed in certain h^+ components. In mutating from an h^- state to an h^+ one other novel characters appeared simultaneously in the same nucleus. Clear examples of this type of correlated mutation may be seen in heterocaryons *A 4-M* where h^- mutated to

h^+, *pan*$^-$ and *A 3-M* where h^- mutated to h^+ *c*, *c* being an auxotroph of unknown type. As well as these associated mutations to h^+ and auxotrophy other less dramatic but nevertheless constant genetic alterations in the normal h^+ appeared in phenotypes of other heterocaryons. These involved temperature sensitivity (*B 5-C*), inhibition by complete medium at 25° and 35° (*A 5-M*), or at 35° only (*A 7-M*, *B 1-M*, and *A 6-C*). Most of these novel h^+ associated phenotypes segregated as if linked to h^-, with the exception of the *pan*$^-$ character of *A 4-M*. In crosses of homocaryotic h^+, *pan*$^-$ with a wild type strain no histidine progeny were detected in approximately 90 spores scored. Although we believe the possibility of an unlinked suppressor type mutation being responsible is unlikely further crossing data are necessary to determine if linked suppressors may offer an explanation of the occurrence of these novel h^+ mutants.

Though the data are not extensive, the probability of these varieties of coincident mutations occurring by two independent events in two sister strands of the same nuclei, seems low. We prefer to conclude that such pairs of genetic mutations resulted from single mutational events in a single nucleus. These observations seem similar to the coincident mutational events Kaplan (1961) has recently reported in *Serratia* and which he has called "switch" mutations.

They are consistent with the sister-strand exchange hypothesis of back-mutation proposed earlier. The central tennet of the hypothesis is the multiple repeat nature of the wild type gene. An independent demonstration that those complex loci in *Neurospora* at which back-mutation occurs are multiple repeats could come from an examination of the structure of the specific proteins produced by them. It may be of significance that the first to be isolated in pure form, glutamic dehydrogenase, appears to be a polymer (Barrat, Fincham, private communication). If we accept the "**central dogma**" that a linear array of bases on the DNA codes a corresponding sequence of amino acids in the protein, we can conceive that a sequence of identical polypeptides in the protein reflects a sequence of identical polynucleotides in the DNA of the gene.

The model of mutation which we suggest (see Fig. 1) explains how small intragenic deficiencies (Ishikawa, 1961) could back-mutate. Those larger than one half of the locus could not back-mutate in one step but might be able to do so by a two-step process by mutating first to an intermediate condition. It also explains how small deletions could occor spontaneously, or if induced by some treatment why this process has "single hit" kinetics.

The sister strand exchange hypothesis is not exclusive of other mechanisms of back-mutation. We can imagine that a single base substitution mutant may back-mutate either by a reverse substitution at a single site, thereby producing an exact wild type back mutant, or by an unequal sister strand exchange which as we have seen may lead to a range of wild-type-like phenotypes.

Summary

To explain the high frequency of partial and intermediate back-mutants and the back-mutation of deletions, a model of mutation production based on unequal sister strand exchange in multi-repeat loci is proposed.

The recovery of complementary products of mutation which the model predicts should be produced from single nuclei is cited as evidence for its plausability.

Back-mutation of the mutant used (a *his-3* mutant, *Gg59*) is associated with a high frequency of simultaneous "switch" mutations of other characters in the same genome.

It is suggested that unequal sister strand exchange may also contribute to the production of spontaneous mutations.

The validity of citing ability to back-mutate as critical evidence for classifying a mutant as a "point" (single base pair substitution ?) mutation is queried.

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THE EFFECTS OF DEUTERIUM OXIDE ON BACTERIA * **

By

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With 2 Figures in the Text

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The replacement of the ubiquitous hydrogen atom by its isotope deuterium produces profound effects in a variety of biological systems (MOROWITZ and BROWN 1953, KRITCHEVSKY 1960). The isotope effects, as predicted by UREY (1933) are probably due to the differences in the mass of hydrogen and deuterium which in turn effect vibrational energy levels and consequently rates of chemical reactions.

Phenotypic and genotypic changes were reported for bacteria (DE GIOVANNI and ZAMENHOF 1959, DE GIOVANNI 1960) and bacteriophage (KONRAD 1960) grown in D_2O-media. The phenotypic effects may be due to altered biochemical relationships caused by the deuterium-containing cell constituents. The genotypic effects may be the result of altered hydrogen bonding in the deoxyribonucleic acid (DNA), specific incorporation of deuterium into sub units of DNA or they may be the result of an indirect effect involving enzymes and precursor pools for DNA systems. All mechanisms may induce the occurrence of errors during DNA replication.

Recently, attention has been focussed on those mutagens whose direct chemical action on DNA has been investigated and interpreted in terms of DNA structure and genetic control. Therefore, the mutagenic action of deuterium is of great interest; it too, should have a direct effect upon the DNA molecule and a correlation between the particular chemical action of deuterium and the genetic consequences observed might help to elucidate further the mechanism of mutation.

In this study, several strains of bacteria were grown in D_2O-media. Phenotypic and genotypic changes occurred and chemical analyses were undertaken in an attempt to correlate the biological observations with the chemical action of the isotope.

Material and Methods

1. Deuterated compounds. The deuterium oxide was supplied as 99.5% D_2O (General Dynamics Corporation) and purified by distillation from alkaline permanganate. Deuterated bases were obtained from the highly polymerized DNA of cells grown in D_2O-media (to be published at a later date).

2. Bacteria. The *Escherichia coli* strains used in this study were: W6; B; B/Sd, a streptomycin-dependent mutant of strain B (strain B/Sd, also referred to as Sd/4, was kindly provided by Dr. M. DEMEREC, Brookhaven National Laboratory); B/S, a streptomycin sensitive

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mutant derived fron B/Sd (this mutant was induced by deuterium): 15 and several mutant derivatives including a thymine-requiring mutant (t^-) described previously as strain I (ZAMENHOF and GRIBOFF 1954) an arginine-requiring mutant (a^- this mutant was induced by deuterium); a histidine-requiring mutant (h_1), this mutant was induced by heat and kindly provided by Dr. STEPHEN ZAMENHOF, a histidine and adenine-requiring mutant ($h^- a^-$); a histidine and guanine-requiring mutant ($h^- g^-$); a histidine and cytosine-requiring mutant ($h^- c^-$) (the last three mentioned strains were kindly provided by Dr. FRANCIS J. RYAN); K_{12} and mutant derivatives including HFr (WOLLMAN, JACOB and HAYES 1956) 1177 F$^-$ and 1817 F$^+$ (LEDERBERG, LEDERBERG, ZINDER and LIVELY 1951). A tryptophane-requiring mutant (tr^-) of *Salmonella typhimurium* referred to as T2YD-10 was also tested (this strain was kindly provided by Dr. FRANCIS J. RYAN). All the above mentioned strains were maintained on nutrient agar slants at 4°. The two *Bacillus subtilis* strains used, 168, an indole-requiring mutant (i^-) and 23 (both strains were kindly provided by Dr. J. SPIZIZEN), were maintained on potato agar slants (SPIZIZEN 1958) and stored at 23°.

3. Media. Nutrient broth, nutrient agar, National Institutes of Health (N.I.H.) agar and Penassay broth were all Difco products. ROEPKE'S synthetic medium (1944) was used for *Escherichia coli*. SPIZIZEN'S synthetic medium (1958) was used for *Bacillus subtilis* and OZEKI'S synthetic medium (1956) was used in the study of *Salmonella typhimurium*. Specific supplements as required were added to the media (10 μg amino acids /ml.; 10 μg purines/ml.; 10 μg pyrimidines/ml.; 10 μg streptomycin/ml. of broth; 50 μg streptomycin/ml. of agar). All types of D_2O-media were prepared by dissolving the dry components into 99.5% purified D_2O.

4. Cell counts. Total cell counts were made by means of a Petroff-Hausser bacterial counting chamber. Viable cell counts were made by plating the cells in or on either nutrient agar or synthetic agar containing specific supplements. The viability refers to the ratio of viable cells to total cells.

5. Growth studies. The responses of strains to D_2O was compared by determining the total cell counts of cultures after 24 and 48 hours of incubation at 37° in various concentrations of D_2O-nutrient broth. The initial cultures contained between 10^2 to 10^3 cells/ml. obtained from an 18 hour control culture.

6. Determination of forward mutations. Cells grown in D_2O-medium as described in No. 5, were diluted and streaked on nutrient agar to detect any mutants of colonial morphology; in addition, auxotrophs were detected by means of a "frog" replica plating technique (ZAMENHOF 1961). Attempts were made to characterize any suspected mutants by restreaking them on nutrient agar, more complex media or on synthetic media containing specific supplements.

7. Back-mutation analysis. *a) D_2O-media.* All starting cultures contained approximately 1×10^5 cells of mutant type/ml. and were incubated and aerated at 37° in H_2O-medium and D_2O-medium for 24 or 48 hours; at the end of the incubation period, the cultures contained approximately $5 \cdot 10^8$ cells/ml. The cells were washed in H_2O-0.85% NaCl (w/v) and immediately plated on the appropriate agars for the determination of the viable cell number and the back-mutant cell number (synthetic agar for auxotrophs; nutrient agar for B/Sd; nutrient agar + 50 μg streptomycin/ml. for B/S). Two to six days after incubation at 37°, the colonies were counted. In all experiments, several induced back mutants were transferred to prove their back-mutant character. Each strain was tested several times and the mutation rates were calculated from the median number of back-mutants obtained from the median number of bacteria plated (LEA and COULSON 1949).

b) D-nitrogenous bases. Parallel 1 ml. cultures (100), started with 10^3 cells/ml. of synthetic-H_2O-medium containing an optimal amount of histidine (10 μg/ml.) and a sub-optimal amount of base (thymine .04 μg/ml. + 0.002% proteose peptone No. 3 (w/v), a Difco product, guanine 0.1 μg/ml. cytosone 0.1 μg/ml. or adenine 0.1 μg/ml.) were incubated at 37°. The effects of normal bases and deuterated bases were compared. After the required incubation time (24—48 hours), the turbid cultures were scored as those in which mutation had occurred and the total number of cells was determined from an average of the total counts from three cultures in the series that had barely visible turbidity ($\pm 1 \times 10^7$ cells/ml.). The Poisson method was used to determine the mutation rate (RYAN and WAINWRIGHT 1954) from base$^-$ to base$^+$. In a slightly modified manner, the mutation rate from h^- to h^+ was determined.

The starting synthetic media contained sub-optimal amounts of histidine (0.1 μg/ml.) and base (0.1 μg/ml.). After 24 hours incubation, at which time all tubes were barely turbid, ($\pm 1 \times 10^7$ cells/ml.) additional normal base was added to give finally an optimal concentration of base (10 μg/ml.). The tubes were scored after 48 hours incubation. The criteria for the occurrence of mutation was the turbid culture; mutation rates were determined as described above. In all cases, the turbid cultures were streaked on appropriate agar to confirm the mutational event; likewise several barely turbid cultures were tested to ascertain the absence of the mutational event.

8. Fluctuation test. The Luria and Delbruck test (1943) was performed to determine whether or not a deuterium-resistant mutant was a spontaneous mutant, or one induced by deuterium. A series of 13 independent 1 ml. nutrient broth cultures and a single nutrient broth culture were incubated for 18 hours at 37°. The starting cultures contained approximately 10^2 sensitive cells/ml. Samples from the individual cultures and samples taken from the single culture were streaked on nutrient agar to determine the viable number and on nutrient agar containing 99.5% D_2O to determine the number of deuterium resistant mutants. The variation in the number of mutants obtained from the independent cultures was compared with that obtained from the single culture.

9. Reconstruction experiments. H_2O-medium and D_2O-medium were inoculated with known mixtures of parent and mutant cells and incubated. The cultures were then diluted and streaked on appropriate agars to determine the proportion of mutant and parent cells.

10. Synchronized culture. B S cells were synchronized by means of a modification of the fractional filtration method described by Maruyama and Yanagita (1956) using 14 sheets of Eaton Dikeman filter paper No. 623, rather than 20 sheets. Total counts were made at 15 minute intervals to ascertain synchrony.

11. Irradiation. Cells grown in nutrient broth were washed in 0.85% (w/v) NaCl, diluted to contain approximately 1×10^8 cells/ml. and 10 ml. portions were placed in a rotating and vibrating petri dish and irradiated with ultraviolet light from a 8 W General Electric Germicidal Lamp G-8, T-5 (2537 A•), at a dose rate of 6.8 ergs/sec./mm^2.

Results

1. Growth. As may be seen in Table 1, D_2O-nutrient broth inhibited the growth of all bacteria tested; the degree of inhibition was strain-specific. As one might expect, the inhibition increased at higher D_2O concentrations and the total cell count increased upon prolonged incubation. In most cases, however, the final cell number was less than that of control cultures after 24 hours of incubation. When bacteria were grown in 99.5% D_2O-medium, there was as much as a five fold increase in the duration of the lag period and a three fold increase in the generation time. The diminished growth in D_2O was also demonstrated on nutrient agar; the diameter of colonies formed was one tenth that of control colonies. The incorporation of 0.5% NaCl (w/v) into D_2O-nutrient broth, enhanced the growth of sensitive bacteria two to one thousand times and bacteria plated on D_2O-nutrient agar, so supplemented gave rise to normal sized colonies. Incubation of cells at 42° had no effect upon their growth in D_2O-media.

Bacteria grown in D_2O-media, for up to 90 hours, showed the same sensitivity to D_2O-media as control cells.

2. Deuterium resistant mutant. The isolation of an apparently spontaneous mutant from strain $15t^-$, more resistant to D_2O-nutrient agar, was achieved. Its colonies on D_2O-nutrient agar contained six times more cells than those of the parent strain and it could be maintained after several transfers on nutrient agar free of D_2O (ca. 200 generations). Strain $15t^-/D$ formed rough colonies on H_2O-nutrient agar; however, other rough *E. coli* strains, did not show a similar resistance to D_2O-nutrient agar. The mutant did not grow as well as the parent in either

Table 1. *The growth response of bacteria to D_2O-media*

Strain	Incub. Time	0 %	50 %	60 %	70 %	80 %	90 %	99.5 %
B	24	***	***	***	***	**	*	-
	48					***	**	*
B/Sd	24	***	*	*	*—			
	48		***	***	***	**	**	*-
B/S	24	***	*	*	* -	—		—
	48		***	***	***	***	**	* -
W 6	24	***	***	**	**	**	*	-
	48			***	***	***	**	*
K 12	24	***	**	**	**	**	**	*
	48		***	***	***	***	***	***
HFr	24	***	***	***	***	**	*	*—
	48					***	***	***
1177 F^-	24	***	*	*	*	—	—	—
	48		**	**	**	*	*	*
1817 F^+	24	***	*	*	*	—	—	—
	48		**	**	**	**	**	*
15	24	***	***	**	*	*	*—	—
	48			***	***	***	***	—
15 t^-	24	***	***	***	***	**	—	—
	48					***	**	—
15 t^+ (t^+)	24	***	***	***	***	***	*	—
	48						***	—
15 h^-a^-	24	***	***	***	***	**	**	—
	48					***	***	**
15 h^-c^-	24	***	***	***	***	**	*	—
	48					***	***	***
15 h^-g^-	24	***	*	*	*—	*—	—	—
	48		***	**	*—	*—	—	—
23	24	***	**	**	*	*	—	—
	48		**	**	*	*	*—	—
168 *i*	24	***	***	***	***	**	*	*
	48					***	**	**
T2YD 10	24	***	*	*	*—	—	—	—
	48		***	***	***	***	**	*—

Growth response code, total cells/ml.
*** $> 10^8$; ** $< 10^8 > 10^7$; * $< 10^7 > 10^6$; *— $< 10^6 > 10^5$; — $< 10^5$.

D_2O-nutrient broth or H_2O-nutrient broth; thus, its resistance to D_2O was demonstrated on agar. In order to be sure that the mutant obtained was a spontaneous one, and not one induced by D_2O, a fluctuation test was performed. The results, as represented in Table 2, show that the mutant isolated was a spontaneous one; the fluctuation of numbers of mutants in the samples of independent cultures was greater than for the samples taken from a single culture. The spontaneous mutation rate from 15 t^- to 15 t^+/D was 1.3×10^{-4}/bacterium/generation; the frequency of mutants was increased 300× by ultraviolet irradation. Deuterium-resistant mutants could not be isolated from any of the other bacterial strains studied.

3. Mutagenic effects. *a) D_2O-media.* For many of the bacterial strains examined, growth in D_2O-medium induced both forward and backward mutations. The forward mutants include stable and unstable auxotrophs. Table 3 gives a listing of some of the forward mutants isolated after growth in D_2O-media; all are nutritional in nature. Stable auxotrophs do not grow on unsupplemented

Table 2. *The fluctuation test analysis of the mutation from deuterium sensitivity to deuterium resistance*

Samples	Average No. mutants per 1×10^4 bacteria plated	Chi-Square	P
Independent cultures . .	4.38	33.25	< 0.01
Single cultures	4.85	11.2	0.6

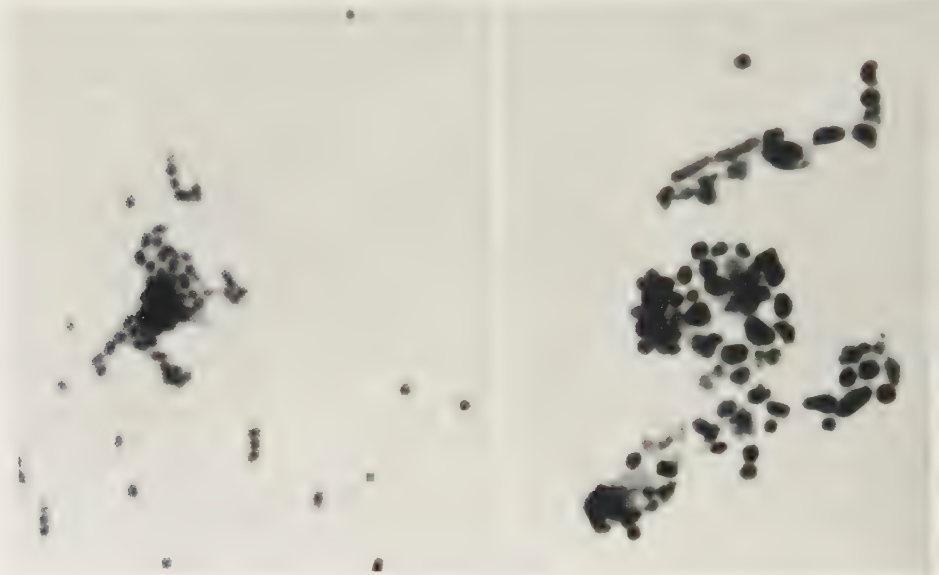
Table 3. *Forward mutants induced by deuterium oxide*

Parent strain	Growth conditions		Mutant	Characteristics	Induced frequency	Back mutation frequency
	hours	media				
15	24	PAD	Stable auxotroph	Arginine requirer	$5/10^{-4}$	$4/10^{-8}$
15 15*t*⁻	18—24	NBD	Small colony	larger colonies formed on NIH or NAS	$1/10^{-3}$	$1/10^{-1}$
15*t*⁻	60	NBD	Unstable auxotroph	methionine requirer	$5/10^{-4}$	$1/10^{-3}$
168*i*⁻	24	PAD	Unstable auxotroph	a) requires a factor in yeast extract	$1/10^{-3}$	$1/10^{-1}$
				b) requires a factor in casamino acids	$1/10^{-3}$	$1/10^{-1}$

Media code: PAD, Penassay broth in 99.5% D_2O; NBD, Nutrient broth in 99.5% D_2O; NIH, National Institutes of Health Agar; NAS, Nutrient agar + 0.5% NaCl (w/v).

synthetic agar (synthetic agar did contain, however, the supplement required by the original strain tested, the auxotrophs obtained required additional supplements, that is they were doubly marked); they revert to the parent type at a low rate. Unstable auxotrophs do not grow on unsupplemented synthetic agar; they revert to the parent type at a high rate. Some mutants formed small colonies on nutrient agar. On N.I.H. agar, a more complex agar, colonies of this mutant are 0.9 the diameter of controls. Also, the addition of 0.5% NaCl (w/v) to nutrient agar improves growth. Upon transfer to nutrient agar or synthetic agar, the small colonies are unstable; the revert to parent sized colonies at a very high rate. The could be maintained as small colonies if early transfers were made. Reconstruction experiments showed that none of the forward mutants had a selective advantage over the parent strains in D_2O-media; the mutants were not selected but were induced by deuterium. Such mutants were not obtained from control cultures. Forward mutations were not studied extensively in all the strains because of the difficulty encountered in their characterization; it was believed that a more quantitative estimate of the mutagenicity of deuterium could be made if its effect upon specific back mutation rates were investigated.

The results, obtained from ten strains grown in D_2O-media (Table 4), show that deuterium effects an increase in the back mutation rate of some but not all of the loci tested. The factor of increase over the spontaneous rate varies from 1.78 to 54.2. In all cases, reconstruction experiments showed that the back-mutants were not selected but were induced by deutrium. The back mutation rate of strains $15a^-$ and $15h_1^-$ were unaffected by deuterium. Three mutants, $15h^-ad$, $15h\ c^-$, and $15h^-g$, all derived from a histidine mutant induced by ultraviolet irradiation showed a small increase in their back mutation rate from histidine to histidine$^+$. *S. typhimurium* grown, in 99.5% D_2O-medium, showed a 10.8 factor



a b

Fig. 1 a and b. Morphological changes in *E. coli B/S* after eight divisions in D_2O-medium. Magnification < 2, 100 for both control cells and deuterated cells. a Control cells (H_2O-medium), b Deuterated cells (D_2O-medium)

increase in its back-mutation rate; this effect was not obtained when the D_2O concentration was reduced to 50%. Deuterium had a greater mutagenic effect on $15t^-$ grown in synthetic-D_2O-medium rather than Penassay D_2O-medium. In synthetic-D_2O-medium the viability of the cells was 0.2%; whereas in Penassay-D_2O-medium, the viability of cells was usually greater than 60%.

B/Sd appears to be most sensitive to the mutagenic action of D_2O-media. The factor of increase over the spontaneous rate was 54.2. All the back mutants obtained are streptomycin independent but sensitive (B S). The B/S strain, induced by deuterium, may also be induced to mutate at a rate *19.5* times higher than the spontaneous rate, upon the cultivation of cells in Penassay-D_2O-medium. The mutants obtained were 65% R (streptomycin resistant and independent) and 35% B/Sd. The B/Sd and B S strains, in this series of experiments, gave rise to "monsters", distorted cells, 2× to 10× larger than controls, that have a 25% viability (Fig. 1).

In an attempt to correlate deuterium mutagenesis with deuterium incorporation, strain B S cells were synchronized by filtration and introduced into Penassay-

H_2O-medium and Penassay-D_2O-medium. The starting cultures contained approximately 1×10^8 cells/ml and were incubated and aerated at 37°. An examination for mutants was made at the end of the first and second divisions and at the end of the 24 hour incubation period. Under these conditions, no increase in the

Table 4. *The effect of deuterium oxide on the back-mutation rates of bacteria*

Strain	Locus	Media[1]	Hours	Mutation rate bacterium/ generation ($\times 10^{-9}$)	Factor of increase over spontaneous rate
168 i^-	$i^- \rightarrow i^+$	PAS	24	1.80	
		PASD	24	3.20	1.78
T2Yd-10	$tr^- \rightarrow tr^+$	PAS	24	0.19	
		PASD	24	2.05	10.80
15 h_1^-	$h_1^- \rightarrow h_1^+$	PAS	24	1.2	
		PASD	24	0.3	0.25
15 a^-	$a^- \rightarrow a^+$	PAS	24	8.8	
		PASD	24	7.6	0.86
15 h^-g^-	$h^- \rightarrow h^+$	PAS	24	18.5	
		PASD	24	44.8	2.40
15 h^-c^-	$h^- \rightarrow h^+$	PAS	24	23.6	
		PASD	24	46.5	1.97
15 h^-ad^-	$h^- \rightarrow h^+$	PAS	24	29.4	
		PASD	24	85.0	2.88
15 t^-	a) $t^- \rightarrow t^+$	PAS	24	8.7	
		PASD	24	110.0	12.50
	b) $t^- \rightarrow t^+$	SM	40	3.9	
		SMD	40	170.0	43.50
B/Sd	Sd → S	PASS	48	1.0	
		PASSD	48	54.2	54.2
B/S	S → Sd ↘ R	PAS	30	1.9	
		PASD	30	37.0	19.5

[1] Media code: PAS, Penassay broth + 0.15% NaCl (w/v) in H_2O; PASD, Penassay broth + 0.15% NaCl (w/v) in D_2O (99.5%); PASS, PAS + 10 μg Streptomycin/ml.; PASSD, PASD + 10 μg Streptomycin/ml.; SM, Synthetic medium + 10 μg thymine/ml. in H_2O; SMD, Synthetic medium + 10 μg thymine/ml. in D_2O (99.5%).

mutation rate occurred. The culture that had been innoculated with a heavy synchronous culture had undergone up to four divisions and had been maintained in the D_2O-medium for 24 hours. The cells of such a culture appeared normal whereas the cells, as described in the previous section, appeared enlarged and distorted after the 24 hours incubation period in D_2O-medium. In the later case, the starting inoculum was low and there were more divisions in D_2O-medium. The total deuterium content of the cells that had undergone three to four divisions was 14.5% and that of cells that had undergone twelve divisions in D_2O-medium was 32.2% (to be published at a latter date).

b) D-bases. Deuterated bases had no effect upon the growth and back mutation rates of specific base-requiring strains (Table 5). The only source of deuterium in the series of parallel cultures, was the deuterium contained in the base. The back-mutation rates, in this series of experiments, were lower than those

obtained in the D_2O-media series and previously described; comparisons may be made however, between the tests containing the normal base and those containing the deuterated base. Further indication that deuterated thymine is not mutagenic rests on the finding that the differences obtained in the back-mutation rate from $15t^-$ to $15t^+$ of cells grown in H_2O-synthetic media as compared to those grown in D_2O-synthetic media were unaffected by the type of thymine supplied.

Table 5. *The effect of deuterated bases on the back-mutation rates of base-requiring strains*

Strain	Locus	Mutation rate in presence of H-base ($\times 10^{-9}$)	Mutation rate in presence of D-base (1×10^{-9})	Factor of increase over spontaneous rate
$15t^-$	$t^- \rightarrow t^+$	0.4	0.4	—
$15h^-g^-$	$h^- \rightarrow h^+$	10.0	13.0	1.3
	$g^- \rightarrow g^+$	14.0	12.0	0.85
$15h^-ad^-$	$h^- \rightarrow h^+$	11.0	9,0	0.82
	$ad^- \rightarrow ad^+$	12.0	16.0	1.3
$15h^-c^-$	$h^- \rightarrow h^+$	13.0	9.0	0.69
	$c^- \rightarrow c^+$	1.7	0.9	0.53

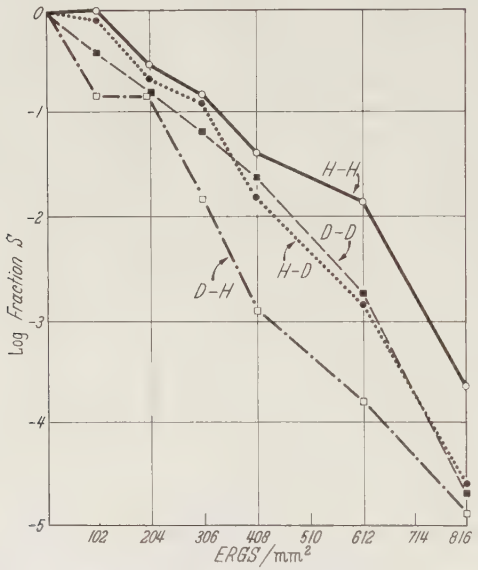


Fig. 2. *Survival of bacteria after ultraviolet irradiation. HH*, grown in H_2O-media, washed in H_2O-0.85 % NaCl (w/v); *HD*, grown in H_2O-media, washed in D_2O-0.85 % NaCl (w/v); *DD*, grown in D_2O-media, washed in D_2O-0.85 % NaCl (w/v); *DH*, grown in D_2O-media, washed in H_2O-0.85 % NaCl(w/v)

4. Sensitivity of deuterated cells to ultraviolet inactivation. Strain 15 was grown in H_2O-medium [Penassay + 0.15% NaCl (w/v)] and D_2O medium [Penassay + 0.15% NaCl (w/v)] for fourteen generations. At the end of incubation, in each case, half the cells were washed and resuspended in H_2O—0.85% NaCl (w/v) and the other half were washed and resuspended in D_2O—0.85% NaCl (w/v). The cells were exposed to low doses of irradiation, diluted and plated in nutrient agar, to determine the number of survivors. A comparison of the survival curves (Fig. 2) for each class of cells, did not show marked differences; of interest, however, is the fact that the most sensitive cells were those that had been grown in D_2O-media and washed and resuspended in H_2O—0.85% NaCl (w/v). They were up to sixty times more sensitive than control cells. The cells that had been grown in H_2O-media and washed in D_2O—0.85% NaCl (w/v) were as sensitive to irradiation as cells kept in D_2O throughout; both classes of cells were up to five times more sensitive than the control cells.

Discussion

The inhibition of growth by deuterium, has been interpreted as being the consequence of an overall cellular effect, resulting from the replacement of hydro-

gen by its isotope deuterium. The mutagenic effect of deuterium may also be due to an overall effect, rather than to the specific incorporation of deuterium into subunits of the DNA molecule.

The isotope effect, evidenced by differences in the reaction rate between labelled and unlabelled compounds, is due to the difference in mass between two isotopes, and is especially marked for hydrogen isotopes. The zero point energy is lower for the deuterium-containing bond which is more stable (Wiberg 1955). The activation energy is greater and the rate of any reaction is correspondingly less for the deuterated compound (Streitwieser 1960). Thus a progressive dissynchrony of general metabolic reactions, including those involved in DNA synthesis, would occur in the deuterated cell. In addition, the geometric configuration of macromolecules, determined by intermolecular hydrogen bonds, and hydrogen containing side groups, would change; the deuterium atom being closer on the average to its bonded atom than its comparable hydrogen atom (Scheraga 1960).

No strain of bacteria was able to overcome the isotope effect and adapt to better growth in D_2O-media; however, Katz (1960), Moses, Holm-Hansen and Calvin (1958) have reported specific patterns of adaptations for some strains of algae. Cultures of algae, grown for long periods of time in D_2O-media and used in the form of a heavy innoculum, grew at a faster rate than cultures that had never been exposed to deuterium. In this study with bacteria, caution was taken to use low innuculum in order that the cells reaction to deuterium be more closely challenged. Under such conditions, in order to reach a maximum growth in D_2O-media, the cells must undergo many divisions, whereas apparent maximum growth would be obtained by fewer divisions if the initial culture contained a large innoculum of cells. Perhaps in the adaptation period for algae, a slightly more deuterium resistant strain was selected that was able to undergo more divisions than the parent strain in D_2O-media. It would appear that, if adaptation to better growth in D_2O-media occurs in bacteria, the improvement is extremely slight. Even the $15t^-/D$ mutant, that was more resistant to D_2O agar, did not show any advantage over the parent strain in any of the D_2O-liquid-media tested.

D_2O has been reported to have slower penetration than H_2O into erythrocytes (Brooks 1935) and to cause plasmolysis of *Nitella* leaves (Brooks 1937). Perhaps osmotic effects are also induced in the bacterial cell by D_2O and sodium chloride has an effect on the permeability of the bacterial cell, helping it overcome some but not all of the growth inhibitory effects of deuterium. The presence of the salt had not effect upon the mutagenicity of deuterium. In the case of algae, the addition of the salt was conducive to growth but had an adverse affect on the adaptation process (Katz 1960).

In view of the higher viscosity of D_2O as compared to H_2O, cultures were incubated at 42^0 in an attempt to equalize the viscosity of the two media (D_2O at 42^0 and H_2O at 37^0). The change of temperature had no effect upon the growth of the bacteria and the indication was that the differences between the viscosity of the media did not account for the differences in growth. Some strains of algae grow better at elevated temperatures while others grow better at lower temperatures (Porter and Weinberger 1954), indicating once more the strain-specific response to growth in D_2O-media.

The distorted cells obtained when B/Sd and B/S were grown for more than eight generations in D_2O-media, were not observed in any of the other bacteria studied. In a similar manner, only some strains of algae tested (KATZ 1960, PORTER and WEINBERGER 1954, MOSES, HOLM-HANSEN and CALVIN 1958); showed an increase in cell size when grown in D_2O-media. It appears that deuterium inhibits one or more stages in cell divisions; indeed, mitotic divisions were inhibited in *Arbacia* eggs incubated in D_2O (GROSS and SPINDEL 1960). An interesting observation of cell distortion was made by BOREK and RITTENBERG (1960) in *Escherchia coli* K_{12} cells, upon the transfer to H_2O-medium of cells of normal size grown in D_2O-medium. Apparently, the mixture of deuterium and hydrogen caused the appearance of distorted cells in this strain, indicating that the effects were not induced solely by the deuterium atom, but to a mixture of the deuterium (in a high proportion) and hydrogen atoms in the cell.

The survival curves of cells exposed to ultraviolet light also indicate that a mixture of isotopes in a cell may be more detrimental than a pure isotope. The cells grown in D_2O and irradiated in the presence of H_2O were most sensitive to ultraviolet irradiation. However, such cells were not as sensitive as cells containing 5-bromouracil in stable configuration in their DNA (GREER and ZAMENHOF 1957). It may well be that exchanges took place when the cells were transferred from D_2O to H_2O and that the DNA containing a mixture of hydrogen and deuterium bonds was less stable to ultraviolet irradiation. The amount of exchange in this case is not known but it is known that exchanges occur and that the rate of exchange is not instantaneous for all the deuterium atoms (LINDERSTROM-LANG 1958). Cell maintained in D_2O throughout, and those grown in H_2O and irradiated in D_2O were equally sensitive to irradiation: thus, the presence of deuterium in labile and non-labile positions in the cell may increase its sensitivity. *Escherichia coli* cells grown in H_2O-media and irradiated in D_2O were three times more sensitive to X-ray irradiation (LASER 1959). This indicated that a cell containing a mixture of isotopes is in a poor state to withstand the irradiation of X-ray or ultraviolet light. The rate of molecular changes induced by such physical agents, may be altered by the presence of deuterium (SHUGAR 1957). DNA containing a mixture of hydrogen and deuterium bonds may be distorted and more easily disrupted by physical forces.

KONRAD (1960) in his mutagenic studies with bacteriophage, was unable to place the mutagenic action of deuterium into either of the classes assigned by FREESE (1959). FREESE believes that one class of phage mutants is induced by the replacement of one purine by another, or one pyrimidine by another, the second class of phage mutants is due to the replacement of a purine by a pyrimidine or a pyrimidine by a purine. By studying which specific loci are induced to mutate, one should be able to characterize the action of a mutagen; however, deuterium induced phage mutants of both clases. This would indicate the non-specific mutagenic action of deuterium. Since deuterium may replace hydrogen atoms in all possible sites, this is not unexpected. The mutagenic action of deuterium does, however, affect some loci and not others and among those that are affected there is a range in the factor of increase over the spontaneous mutation rates.

In this study, a histidine mutant induced by heat, showed no increase in the back-mutation rate after growth in D_2O-medium but the histidine and nitrogenous base-requiring mutants, all derivatives of a histidine requiring mutant induced by ultraviolet irradiation, showed a two to three fold increase in the back mutation rate from h^- to h^+. It is very likely that the two histidine mutants differ in their specific site of injury and that in one case the specific action of deuterium causes a change and induces a reversion, while in the other case it does not. Another interesting observation is that an arginine-requiring mutant induced by deuterium could not be made to revert by deuterium. The mechanism by which deuterium produced the forward mutation did not operate in inducing a reversion to the parental type.

In the study with the streptomycin dependent mutant (B/Sd), both spontaneous and induced back mutants were all streptomycin independent but streptomycin sensitive (B S). This supports the findings of the reconstruction experiments, namely that the back mutant did not have a selective advantage. If the back mutant appeared in the culture tube containing streptomycin, it would be eliminated because of its sensitivity to streptomycin. Furthermore, the results indicate that the majority of back mutants observed, probably appeared on the assay plate containing no streptomycin, when the deuterated cell started to divide. Washing the control cells in D_2O or the deuterated cells in H_2O, in an attempt to allow for some isotope exchange, had no effect upon the mutant yield. The exchange attempted was probably not equal to the changes that take place when the deuterated cell divides on H_2O-agar.

For the mutant B S, 70% of the spontaneous back-mutants were B/Sd and 30% were independent but resistant (R); this is comparable to what was described by Bertani (1951). Among the back-mutants induced by deuterium, about 35% were streptomycin resistant but dependent and 65% were streptomycin resistant and independent. It appears that the spontaneous event of back mutation leads to a somewhat different proportion of mutant types from those induced by deuterium. If the intact gene gives the phenotype of streptomycin sensitivity, then the alteration of any site may give rise to the streptomycin resistant phenotype; depending upon where the alteration takes place, however, the mutant may be streptomycin resistant and dependent or streptomycin resistant and independent. If the differences between the percentages of mutant types obtained spontaneously and those induced by deuterium be considered significant, one might suggest that the mutants appearing after deuterium exposure resulted from an event other than the spontaneous one, and that the mutagen was acting in a specific way.

The mutagenic action of deuterium does not appear to depend upon the deuteration of the nitrogenous bases in the DNA molecule. The bases tested contained deuterium in the stable positions. If the methyl group at the C_5 in thymine contained deuterium, one might expect that there would be a change in the size of the group due to the shorter bond distance between deuterium and carbon than hydrogen and carbon and that this would induce changes in the cell as did 5-bromouracil in bacteria (Zamenhof, de Giovanni and Greer 1958, Rudner and Baibinder 1960) and bacteriophage (Litman and Pardee 1956,

BENZER and FREESE 1958). This was not the case; apparently the substitution of deuterium for hydrogen in the nitrogenous base has less of an effect than the replacement of a methyl group by a bromine atom. Even cytosine that had both non-labile hydrogens replaced by deuterium did not induce any changes in the strain that required cytosine for growth. The effect of deuterium replacement of labile hydrogens in the bases, by itself, however, can not be evaluated.

In one strain examined (B/S), mutagenesis was not obtained even after four divisions and 24 hours incubation in D_2O-medium (large inoculum). When the strain was examined after eight divisions in D_2O-media (small inoculum), however, mutants were found. Because of the isotope effect, the incorporation of deuterium by the cell probably does not occur at the same rate as hydrogen; rather, there is a preferential uptake of hydrogen and only after the cell has undergone many divisions does the incorporation of deuterium into the cell become significant. Indeed, PORTER and WEINBERGER (1954) found a preferential uptake of hydrogen in early divisions of algae in D_2O-media. The maximum effects induced are probably the result of much incorporation of deuterium into cell constituents, with the result that the steady state of the cell's metabolism is upset. The mutation probably occurs when a highly deuterated cell is transferred to H_2O-medium and starts to divide. It has been seen that an isotope mixture is harmful to cells, especially when the deuterium percentage is high. As the deuterated cell starts to exchange and to synthesize new components in the H_2O assay agar, unsynchronized metabolic rates may give rise to distorted cells that reflect general disorder. Interference with synthetic pathways involved in DNA synthesis, plus distortion of the DNA molecule, may lead to the induction of errors during replication and to permanent changes in the genetic makeup of the cell. Whether the final effect is due to a specific reaction altered by the isotope, remains to be elucidated. Perhaps in the future, the availability of deuterated cell constituents will enable one to discover what key metabolic reactions trigger the mutagenic effect shown by high concentrations of deuterium in the cell.

Summary

The growth of several strains of bacteria was inhibited by D_2O-media. The degree of inhibition was strain specific. The incorporation of 0.5% NaCl (w/v) to the D_2O-medium decreased the inhibition of growth. No adaptation to better growth in deuterium was obtained in any of the strains tested; however a deuterium resistant mutant was obtained from one strain. Two strains tested formed enlarged, distorted cells when grown for more than eight generations in D_2O-media. Cells grown in D_2O-media and washed and resuspended in H_2O—0.85% NaCl (w/v), were up to sixty times more sensitive to ultraviolet irradiation than control cells. Deuterium induces the occurrence of forward mutants in some strains. Many loci, but not all, showed an increase in the back-mutation rate over the spontaneous level, indicating some specificity of action by deuterium. Deuterated bases, obtained from a strain grown in D_2O-medium, did not induce any phenotypic or genotypic effects when supplied to specific baserequiring strains. The amout of deuterium incorporated into the bacteria may be related to the mutagenic effect induced. The genotypic and phenotypic effects induced by deuterium are probably the result of an overall isotope effect.

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A METHOD FOR SECURING THYMINELESS MUTANTS FROM STRAINS OF *E. COLI** **

By

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With 6 Figures in the Text

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Thymineless mutants are important and useful for genetic and biochemical research because thymine is found in DNA and nowhere else. *Thy*$^-$ mutants of *Escherichia coli* are, however, difficult to obtain and have been found only in *E. coli* 15 (Cohen 1954 and Barner 1954) and *E. coli* B_3 (Brenner 1959), perhaps because the penicillin-screening technique ordinarily used eliminates them through the thymineless death (Cohen 1954) which takes place in medium lacking thymine. Recently Kaudewitz (1959) found that, after treatment of *E. coli* B with nitrous acid at a low pH without penicillin screening, one percent of the surviving cells were auxotrophic mutants; among 344 of these he found 2 *thy*$^-$ mutants. This method is, however, laborious.

The authors have discovered a method by which *thy*$^-$ mutants can be obtained easily (Okada, Yanagisawa and Ryan 1960). The experiments were performed with the techniques used by Freese (1959) for phage. Nitrous acid, proflavine, and 2-aminopurine were being used to try to increase the frequency of mutation from *his*$^-$ to *his*$^+$. This mutation rate was not changed, but, rather surprisingly, many *thy*$^-$ mutants were found among cells treated with aminopterin (AM), 5-bromodeoxyuridine (BUDR), and thymidine (Td).

This paper presents the technique, and suggests the mechanism, by which the *thy*$^-$ mutants were produced.

Materials and Methods

Strains. In most experiments, *E. coli* 15 *his*$^-$*col-r* (histidineless, colicine-resistant; Ryan, Fried and Mukai 1955) and the parent strain, *E. coli* 15 *his*$^-$*col-s* (histidineless, colicine-sensitive), were used. The colicine-sensitive strain is sensitive only to phage T 2, and the resistant one, to all T phages (Mukai 1960). These markers were used to check for contamination. *E. coli* 15 *his*$^-$*met*$^-$*col-s* (histidineless, methionineless, colicine-sensitive) was used for the reconstruction experiments. Several derivatives of *E. coli* K 12 were also used; they are listed in Table 1.

Media. The minimal medium used was a modified Gray and Tatum (Ryan and Schneider 1949) — Table 2 — with 1.8% Difco agar added when desired. The medium was made

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** We dedicate this paper to Professor L. C. Dunn, who retires at the end of this academic year after 34 years of service to Columbia University, with the wish that he will long continue his contributions to genetics.

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up to a 20-fold strength, but $MgSO_4 \cdot 7\ H_2O$, $CaCl_2$ and trace elements were not included. For use the medium was diluted and $MgSO_4 \cdot 7\ H_2O$, $CaCl_2$ and trace elements were added. The sugar, or sugar and agar, solutions were sterilized separately and added to the mineral solution. The concentration of sugar (glucose) was 0.05% in liquid medium and 0.5% in

Table 1. *Derivatives of E. coli* K 12

Mating Type	Strains	Genetic Character
Hfr	Hfr(CS101)	*met⁻ str-s* T2-*r* T3-*r*
	Hfr(H)	*met⁻ str-r*
	Hfr(Prot)	Prototroph *str-s*
F^+	Wild type K12	Prototroph
	CR16	*his⁻*
F^-	W-1177	*thr⁻ leu⁻ B₁⁻ str-r* T1, 5-*r* T6-*r*
	CR34(C600)	*thr⁻ leu⁻* T1, 5-*r lac⁻*

Table 2. *Composition of modified* Gray *and* Tatum *medium*

Medium		Trace Elements	
NH_4Cl	5.0 g	$Na_2B_4O_7 \cdot 10\ H_2O$	176 mg
NH_4NO_3	1.0 g	$CuSO_4 \cdot 5\ H_2O$	786 mg
Na_2SO_4	2.0 g	$Fe_2(SO_4)_3 \cdot 6\ H_2O$	1.82 g
$MgSO_4 \cdot 7\ H_2O$	0.1 g	$MnCl_2 \cdot 4\ H_2O$	144 mg
$CaCl_2$	ca. 1 mg	$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	73.6 mg
K_2HPO_4	3.0 g	$ZnSO_4 \cdot 7\ H_2O$	17.62 g
KH_2PO_4	1.0 g	Distilled water	2000 ml
Trace elements	1 ml	p_H: brought to ca. 2.0 with 5 ml conc. HCl	
Distilled water	1000 ml		
Sugar 0.05% in liquid medium 0.5% in agar medium			

agar medium. Other supplements used were: L-histidine monohydrochloride (H), 25 μg/ml; DL-threonine (Thr), 200 μg/ml; thiamine hydrochloride (B_1), 0.02 μg/ml; thymidine (Td), 20 μg/ml; DL-leucine (L), 20 μg/ml; thymine (T), 40 μg/ml; and DL-methionine (M), 20 μg/ml, added as necessary for the nutritional mutants used. The thymine was secured from the Nutritional Biochemicals Corporation and the thymidine and aminopterin from the California Foundation for Biochemical Research.

G and T medium was supplemented with 12 of the 14 substances whose production is assumed to be inhibited by AM. Vitamin B_{12} and thymidine were omitted for no interesting

Table 3. *Supplements in G and T*(12) *medium*

	μg ml		μg/ml
Glycine	20	Uracil	5
DL-methionine	40	Deoxycytidine	10
DL-leucine	20	Ca pantothenate	0.2
L-valine	10	Thiamine HCl	0.2
DL-serine	20	Pyridoxine	0.2
Adenine	10	L-histidine HCl	25
Guanine	10		

reason. The 12 substances in this G and T(12) medium were the same as those used by Freese (1959) and are listed in Table 3. In addition to these, histidine (25 μg/ml) was always added when his⁻ strains were used.

The complete medium used was YECA agar which is G and T medium supplemented with 0.5% Difco Yeast Extract, 0.5% Sheffield NZ-case, and 2% agar. To this medium

20 μg/ml of Td were always added to support the growth of thy mutants. The latter medium is called YECA(Td).

Methods. A freshly-grown colony of *E. coli* 15 *his col-r* was inoculated into 5.0 ml of G and T(12) medium and incubated at 37° C with aeration for 15—16 hours; in this time the culture reached full turbidity, about 10^9 cells/ml. This culture was washed twice with saline and resuspended in the same volume of saline. This procedure was always followed before starting an experiment.

First, this suspension was diluted to 10^{-1} and 0.1 ml was put into a mixture of 4.5 ml of G and T(12) medium with 0.5 ml of 2 mg AM/ml (final concentration 200 μg/ml). This is point A of Fig. 1. The mixture was incubated at 37° C with aeration, to make the cells biochemically thymine-deficient. After 24 hours' incubation, 0.5 ml of Td, Td + BUDR, or BUDR alone (each at 2 mg/ml), were added. Controls received no addition. All cutures were adjusted to 6.1 ml with sterile distilled water. This is point B in Fig. 1. The cultures were then incubated further at 37° C. After 48 or 72 hours' incubation, cultures lacking Td were not turbid; complete growth was obtained only with Td, or Td + BUDR. These turbid cultures were diluted and plated on both YECA and G and T agar to determine the total viable cell count and the number of *his*$^+$ mutants. This is point C of Fig. 1.

Results

a) Discovery of the thymineless mutants. The proportion of *his*$^+$ mutants in the population obtained by the method described was not increased, but after several days at room temperature, many unusual thin colonies were found on YECA agar plated with the full-grown cultures. These colonies proved to be sensitive to all T phages, like the original *E. coli* 15 *his*$^-$*col-r* strain. When examined for their nutritional requirements, they were found to be *thy*$^-$. These mutants did not require any other novel substances, and their original markers (*his*$^-$ and sensitivity to T phages) were unchanged; as is true with most samples of mutants, some are more stable than others. No other nutritional mutants were found in these populations. The results indicated that *thy*$^-$ mutants could be obtained without using the mutagen, BUDR, which was thereafter omitted to simplify the procedure.

Curiously, the *thy*$^-$ mutants do not grow well on YECA agar supplemented with *T*, but they do well with Td. The mutants grow, however, on G and T medium supplemented with either T or Td. For further experiments, therefore, 20 μg Td/ml were added to the YECA agar medium. One *thy*$^-$ mutant was found to die in G and T medium without thymine, but not as fast as Cohen and Barner's *thy*$^-$ mutant.

The production of *thy*$^-$ mutants by this method is highly reproducible when appropriate chemical reagents are employed. The question arises whether the appearance of *thy*$^-$ mutants is due to spontaneous mutation and selection, induction of mutation, or to both induction and selection. Experiments have been performed to check these three possibilities.

b) Examination of the mutagenic action of AM. To determine the frequency of *thy*$^-$ mutants in the population at points A, B, and C (Fig. 1), bacterial suspensions (ca 10^9/ml) were diluted and plated on YECA(Td) agar so as to give 50—100 colonies per plate. When colonies appeared they were replicated onto G and T(H) and G and T(HT) agar.

As shown in Fig. 1 the number of bacteria was reduced to 10^{-3}—10^{-4} during treatment with AM in G and T(12) medium for 24 hours at 37° C. The frequency of *thy*$^-$ mutants in the population at points A and B was less

than 0.1 percent, although the number of bacteria at point B had decreased to 10^2—10^3/ml. At point C, however, 20—80 percent of the cells were *thy*⁻ mutants (Table 4). These results do not appear to indicate that AM has a mutagenic effect by itself.

Table 4. *The frequency of thymineless mutants in a population of E. coli* 15 *his⁻col-r at points A, B, and C*

Exp. No.	Points	Total No. of colonies	Total No. of *thy*⁻ mutants	Frequency (%) of *thy*⁻ mutants
1	A	1252	0	< 0.079
	B	1248	0	< 0.080
	C(Td)	522	279	53.5
2	A	3112	0	< 0.032
	B	1098	0	< 0.091
	C(T)	50	13	26.0
	C(Td)	97	23	23.7
	C(Td + BUDR)	193	157	81.3

The cell suspensions at points A, B, and C were diluted and plated on YECA(Td) agar and the resulting colonies were replicated onto G and T(H) and G and T(HT) agar. The substances added at point B in each experiment are indicated in parentheses in the second column.

c) Examination of the selective action of AM. To determine whether there was any selective action of AM favoring *thy*⁻ mutants, freshly-prepared cell suspensions of *E. coli* 15 *his⁻met⁻col-s* and *his⁻thy⁻ col-s* were mixed together in equal proportions; 0.1 ml of this mixture was put into 5 ml of G and T(12) medium, both with and without AM. The tubes were incubated at 37° C and aliquots were taken from the mixtures at various intervals and plated on G and T(HM) and G and T(HT) to determine the number of viable cells of each type. The *his⁻met⁻* strain was chosen because it could easily be selected for with appropriate media.

Fig. 2 shows that the *thy*⁻ cells die more rapidly than *thy*⁺ in both mixtures. Thus we conclude that there is no selective action for *thy*⁻ on the part of AM alone.

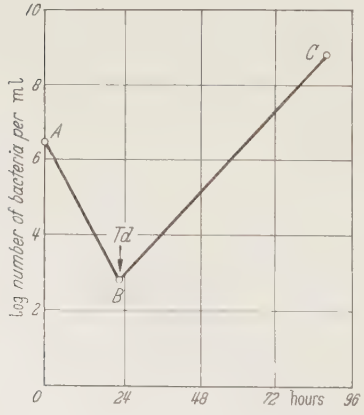

Fig. 1

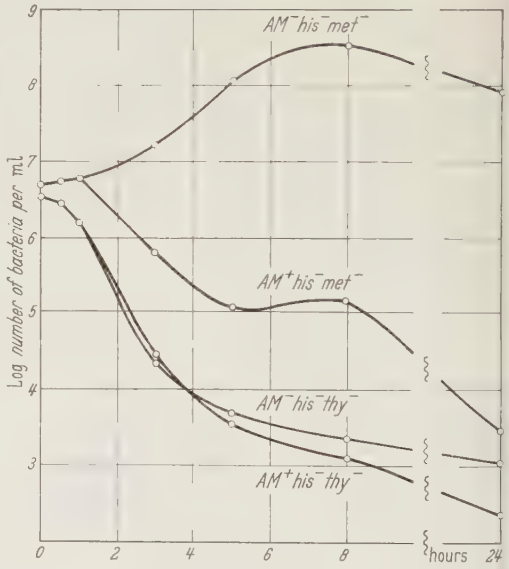

Fig. 2

Fig. 1. The growth of *E. coli* 15 *his⁻col-r* in medium containing aminopterin to which thymidine (*Td*) is added after 24 hours

Fig. 2. Survival of *his⁻met⁻thy⁺* and *his⁻met⁺thy⁻* strains of *E. coli* in media with (*AM⁺*) and without (*AM⁻*) aminopterin

d) Washing out experiment. To determine the effect of AM on the production of *thy*⁻ mutants between points A and B, the cells were washed twice with saline at point B and resuspended in G and T(12) containing 200 μg Td/ml in one series, and the same amounts of Td and AM as a control. Cultures lacking AM became turbid after 24 hours' incubation at 37° C, and those with AM and Td usually reached full turbidity after 48 hours. In the former, no *thy*⁻ mutants could be found, even after 48 hours at 37° C. *Thy*⁻ mutants were found, however, in the other series. From this we conclude that AM is not active during the A to B period, but is important in the B to C period.

e) Experiments without aminopterin pretreatment. To attempt to clarify our findings, a series of experiments was started at point B. Cells grown overnight in G and T(12) were diluted to 10^{-4} and 0.1 ml of the culture was added to 5 ml of minimal medium with various combinations of AM, Td, and BUDR, as shown in Table 5. After 48 or 72 hours at 37° C the cultures became fully turbid and

Table 5. *The composition in* ml *of solutions used to increase the frequency of thy⁻ mutants*

Exp. No.	Bacterial suspension (10^{-4} dil.)	G and T (12)	AM (2 mg/ml)	Td (2 mg/ml)	BUDR (2 mg/ml)	Dist. H_2O	Total volume
1	0.1	4.5	0.5	0.5	0.5	0	6.1
2	0.1	4.5	0.5	0.5	—	1.5	6.1
3	0.1	4.5	—	0.5	—	1.0	6.1
4	0.1	4.5	—	0.5	0.5	0.5	6.1

thy⁻ mutants were in some cases found in a frequency of more than 10 percent (Table 6). If AM was omitted, it took just 24 hours to reach full turbidity and no *thy*⁻ mutants were found, even when the cultures were allowed to stand for 48 or 72 hours. This demonstrates conclusively that the AM treatment is not necessary during the A to B period, but it *is* necessary during the B to C period for the production of *thy*⁻ mutants. In addition, BUDR seems to increase the frequency of *thy*⁻ mutants.

Table 6. *The proportion of thy⁻ mutants found at point C in the cultures with various combinations of Td, AM, and BUDR*

Exp. No.	AM	Td	BUDR	No. of colonies	No. of *thy*⁻ mutants	% of *thy*⁻ mutants
1	+	+	+	137	52	38.0
2	+	+	—	132	12	9.1
3	—	+	—	165	0	$<$ 0.61
4	—	+	+	163	0	$<$ 0.61

f) Three-tube experiments. To discover whether the *thy*⁻ mutants appearing at point C pre-existed in the population at point B or were produced during this incubation period, the following experiment was performed. From a freshly-prepared cell suspension, samples were diluted and plated on YECA(Td) agar; the resulting colonies (3×10^3) were tested for their thymine requirement. If none are found, the proportion of thy⁻ mutants must be less than 1 per 3000 (<0.03 percent). Suppose the cell suspension was distributed among three cultures, so that each received about 30 cells, and that these were incubated

at 37° C. Since the probability of introducing a *thy*⁻ mutant into one culture of three is less than 30/3000 ($<10^{-2}$), into two cultures it is $<10^{-4}$, and into all three, $<10^{-6}$. This means that the *thy*⁻ mutants appearing at point C were produced during the growth in G and T(12) medium containing AM and Td, and did not pre-exist at the starting point, B. This is the logic.

Table 7. *Three-tube experiments to determine the cause of the change in frequency of thy⁻ mutants from point B to point C after the addition of Td*

Exp.	No. of culture	No. of cells per culture	No. of colonies	No. *thy*⁻ mutants	Chance of introducing a pre-existing *thy*⁻ mutant into 3 cultures
			Point B		
A	3	3.8×10^{1}	3459	0 (<0.029%)	10^{-6}
B	3	4.5×10^{2}	4157	0 (<0.024%)	10^{-3}

Exp.	No. of cultures	No. of cells per culture	No. of colonies	No. *thy*⁻ mutants	% of *thy*⁻ mutants
			Point C		
A	1	3.2×10^{8}	625	4	0.64
	2	2.0×10^{8}	408	0	<0.25
	3	4.3×10^{8}	862	51	5.9
B	1	2.2×10^{9}	1100	977	83.8
	2	9.2×10^{8}	458	287	62.7
	3	1.2×19^{9}	583	379	65.0

Table 7 shows that *thy*⁻ mutants were obtained in two cultures out of three in experiment A, and in all three cultures in experiment B, although no *thy*⁻ mutants were found at starting point B. Thus it is concluded that the *thy*⁻ mutants obtained arose during the growth of the parent strain, starting at point B.

g) The frequencies of thy⁻ mutants at various periods during growth. The next problem to be considered was the manner of appearance of *thy*⁻ mutants during the treatment. Aliquots were taken from the culture at various intervals during the growth period (B to C) and each sample was checked, both for total viable cell number and for the number of *thy*⁻ mutants (Fig. 3). The usual G and T(12) medium containing AM and Td was supplemented with BUDR, because this chemical seemed to increase the frequency of *thy*⁻ mutants, and it was believed it would enable the discovery of the mutants before the culture reached full growth.

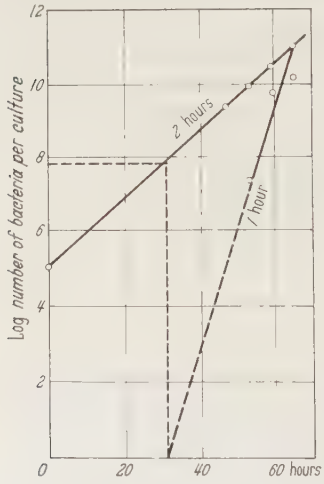

Fig. 3. Generation time of *E. coli thy*⁺ (2 hours) and of *thy*⁻ mutants (1 hour) in medium containing AM, Td and BUDR. Extrapolation to the abscissa allows an estimate of the minimum population in which one *thy*⁻ mutant may be found

From the slope obtained (Fig. 3) the generation time of *E. coli* 15 *his⁻col-r* is calculated to be about 2 hours, and that of the *thy*⁻ mutants, about 1 hour. If the curve representing the new *thy*⁻ mutants is extrapolated to zero along the abscissa, we can estimate the size of the population in which one *thy*⁻ mutant

may be found. Fig. 3 shows that this is about 3.4×10^7/ml. This is a reasonable population size for the presence of mutants in *E. coli*.

h) The effect of cell concentration. If the spontaneous mutation and selection hypothesis were correct, *thy*⁻ mutants might be obtained even if treatment started at a concentration of 10^7 cells/ml and the incubation time shortened to 24 hours, because the inoculum might be large enough to contain *thy*⁻ mutants but small enough so that they would be selected for during its growth. If the inoculum contained 10^8 cells or more, enough growth may not ensue for mutants to be selected for and they would be in too low frequency to be found. However, if the induction and selection theories were correct, treatment with AM and

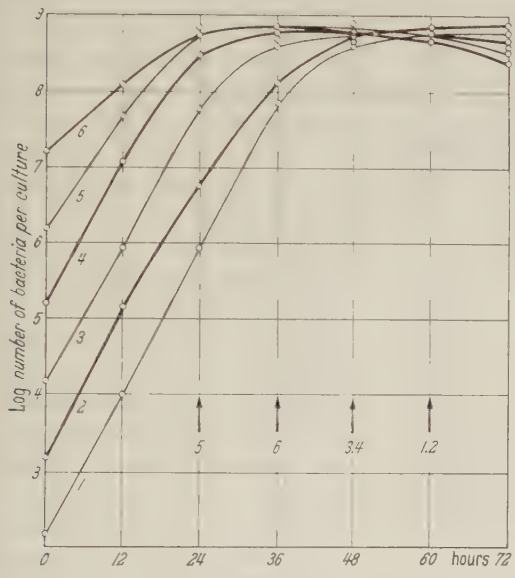

Fig. 4

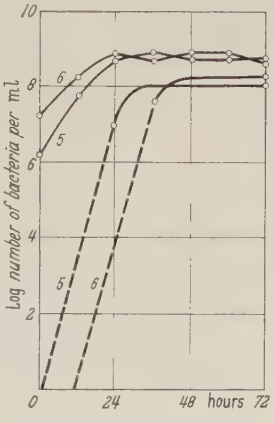

Fig. 5

Fig. 4. Growth curves of *E. coli* 15 *his*⁻*col-r* started with inocula of various size from point B. The numbers and arrows indicate the culture and the point at which *thy*⁻ mutants are first found

Fig. 5. Growth curves for cultures of *E. coli* 15 *his*⁻*col-r* started with 10^6 cells/ml and 10^7 cells/ml at point B. The lower curves represent the number of *thy*⁻ mutants in each. Extrapolation was made to the abscissa on the assumption of a generation time of one hour

Td for 48 hours might be necessary to produce mutants and then even a small culture may not produce *thy*⁻ mutants at an appreciable rate after 24 hours' incubation, in spite of having attained full turbidity.

Six different concentrations of cells were used for the experiments, as shown in Fig. 4. After 24 hours' incubation, *thy*⁻ mutants were found in suspension 5 which started with about 10^6 cells/ml, and after 36 hours, in 6, which was started with about 10^7 cells/ml. After longer intervals, mutants were also found in suspensions 1—4. The concentrations used in suspensions 5 and 6 were chosen for further examination of the hypothesis of mutation and selection.

Fig. 5 shows that, assuming a generation time of 1 hour, *thy*⁻ mutants can be produced during the incubation period. In the experiments in which concentrations of 10^8 and 10^9 cells/ml were used at starting point B, no *thy*⁻ mutants were found. Thus there is no evidence to contradict the spontaneous mutation and selection hypothesis.

i) Reconstruction experiments. To perform reconstruction experiments, *thy*⁻ mutants were obtained from both *E. coli* 15 *his*⁻*met*⁻*lac*⁻*col-s* and *his*⁻*col-s* strains. The combinations are *thy*⁺*met*⁺*lac*⁺ and *thy*⁻*met*⁻*lac*⁻ and *thy*⁺*met*⁻*lac*⁻ and *thy*⁻*met*⁺*lac*⁺. The met⁻lac⁻ markers are combined with the *thy*⁻ marker in one group, and with *thy*⁺ in the other. In this way any possible effect of *met* or *lac* on the growth rate can be ruled out. G and T(12) medium containing AM and Td was used.

The results shown in Fig. 6 support the hypothesis of spontaneous mutation and selection, because in both combinations *thy*⁻ mutants always grew faster than *thy*⁺ strains. Growth rates were about the same as those found in the experiment in which the frequencies of *thy*⁻ mutants were checked at various intervals during the growth period.

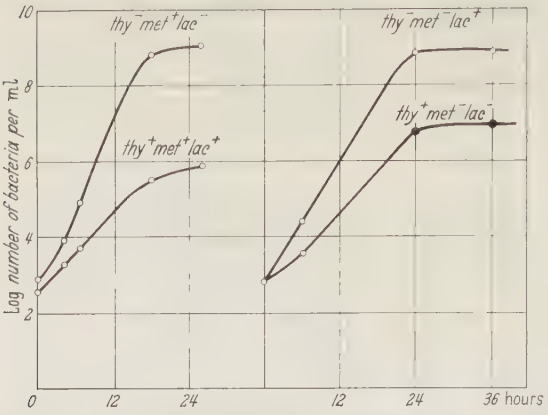

Fig. 6. A reconstruction experiment comparing the growth rates of marked strains of *E. coli* in the presence of AM and Td. The black circles represent estimates of the maximum number of *thy*⁺ cells that could have been present and been undetected by plating the dilutions employed

j) Other bacteria. The AM-Td technique for producing *thy*⁻ mutants was tried with *E. coli* K12 and *Salmonella typhimurium*. The *E. coli* strains were a prototrophic revertant of HfrH, K12 wild type, W-1177 and CR 34, whose genetic characters are listed in Table 1. These strains, except for HfrH (prot.), are usually difficult to grow in G and T(12) medium. *Thy*⁻ mutants, however, could be obtained in high frequencies, so that we assume a mutation which allowed the cultures to grow well in this medium. A few of the K12 *thy*⁻ mutants (in strains HfrH and W1177) were examined for survival in medium without thymine; they did not die but rather synthesized a little DNA and grew to a slight extent.

No *thy*⁻ mutants were obtained from *Salmonella typhimurium*.

k) Mechanism of AM-Td interaction. It is difficult to determine why *thy*⁻ mutants grow faster than *thy*⁺ in the presence of AM and Td. One possibility is that there is a difference in the rate of incorporation of thymidine into DNA.

Further study of this question has been carried out using thymine-2-C^{14} in cooperation with I. Matsumoto at Kanazawa University, and also at Columbia University in a preliminary way. Thymine-2-C^{14} (1 μg/ml, activity 30 μc/ml) was added to the culture in place of unlabelled thymine; aliquots were taken at once and after 5 minutes. The cells were washed once with G and T medium containing 10 μg Td/ml, and the radioactivity of the PCA-soluble and -insoluble fractions of the cells were checked. It was found that, 5 minutes after adding the hot thymine, both acid-soluble and -insoluble fractions of the *thy*⁻ mutants incorporated the radioactive substance much faster than those from the *thy*⁺ cells. This seems to suggest that the two strains differ in permeability or reten-

tiveness inasmuch as the *thy*⁻ mutants take up and incorporate thymine more easily than *thy*⁺ cells. An experiment to check this hypothesis is planned.

Discussion

Because BUDR is a thymine analogue and thymine seems to be incorporated more readily by *thy*⁻ mutants than by *thy*⁺ strains, it is believed that BUDR would also be incorporated more readily by the *thy*⁻ cells. The rate of growth of the *thy*⁻ mutants is not inhibited by BUDR; indeed, it may be slightly increased by BUDR in the presence of thymine, as attested by data showing that BUDR has the effect of enhancing selection. Thymidine can be replaced by thymine in the culture medium used to obtain *thy*⁻ mutants.

Sometimes cultures grow from point B to full turbidity after 24 hours' incubation at 37° C in G and T(12) containing high concentrations of AM and Td without containing any *thy*⁻ mutants. When these rapidly-grown cells are isolated and purified and then incubated again in the usual medium containing AM, they are found to grow to full turbidity once more after 24 hours at 37° C. They are AM-resistant in the sense that they grow at the usual rate in G and T(12) medium containing high concentrations of AM and Td.

We now have two kinds of strains which can grow normally in medium containing AM. One is *thy*⁻ and the other, *thy*⁺. Possibly our *thy*⁻ strains arise as mutations in the AM-resistant *thy*⁺ strain. This is, however, unlikely, for it would involve a two-step mutation which would be too rare and their selective advantage would not be unique. *E. coli* 15 *thy*⁻ (Cohen's strain) grows fast in AM: *thy*⁻ and AM-resistance seem to be correlated expressions of the same gene.

We believe that no *thy*⁻ mutants of *Salmonella typhimurium* were obtained because these bacteria are not sensitive to AM and grow at about the usual rate in its presence without thymine. Selection for the *thy*⁻ character is therefore negligible under the condition of this experiment.

In order to obtain mutants, the basic procedure is to suppress the growth of *thy*⁺ cells but not of *thy*⁻. If similar methods can be used to select for other mutants, we will be able to obtain them at will.

Conclusions

1. A method for obtaining thymineless mutants is described; it is based on the selective growth of bacteria in medium containing high concentrations of aminopterin and thymidine.

2. The forces which allow the appearance of the *thy*⁻ mutants seem to be spontaneous mutation and selection. The selective agent for *thy*⁻ mutants is aminopterin, which depresses the growth rate of the *thy*⁺ cells but not that of the *thy*⁻ bacteria in the presence of thymidine.

3. *Thy*⁻ mutants can be obtained from various derivatives of *E. coli* 15 and K 12.

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β-GALACTOSIDASE SYNTHESIS BY *ESCHERICHIA COLI* ZYGOTES IN THE ABSENCE OF DNA SYNTHESIS* **

By

KEIKO YANAGISAWA*** and DAISUKE NAKADA

With 1 Figure in the Text

(Received July 24, 1961)

Transfer and expression of the genes responsible for the synthesis of β-galactosidase have been studied extensively in the mating system of *Escherichia coli* K 12 (PARDEE, JACOB and MONOD 1959; PARDEE and PRESTIDGE 1959; RILEY, PARDEE, JACOB and MONOD 1960). One of the most important conclusions brought out by these studies is that the enzyme is actually synthesized by the merozygotes before the genetic determinants contributed by the Hfr donor parent are integrated into recombinant chromosomes. In other words, the genetic material is able to function in the zygotes (phenotypic expression) independent of any phenomenon of recombination between genetic materials.

The experiment described in this paper further supports this conclusion by showing that the genetic material can function in the zygotes in the absence of DNA synthesis. In order to block DNA synthesis, 5-fluorodeoxyuridine (FUDR, a gift from HOFFMANN-LA ROCHE Inc.) was added to the mating medium and thus a thymine-deficient condition was created (COHEN, FLAKS, BARNER, LOEB and LICHTENSTEIN 1958; LORKIEWICZ and SZYBALSKI 1960).

The members of the mating pair were both *E. coli* K 12. The Hfr H strain carried the alleles z^+ (β-galactosidase positive), i^+ (inducible for this enzyme), and Sm^s (streptomycin sensitive). The F^- W 1177 strain carried z^-, i^-, Sm^r, thr^- (threonineless), leu^- (leucineless) and B_1^- (vitamin B_1-less) alleles.

Exponentially growing cells of both Hfr and F^- strains in glycerol-salts medium (M 63; PARDEE, JACOB and MONOD 1959) with necessary supplements were collected and washed by centrifugations. Mating was performed at 37° C in the absence and presence (1×10^{-4} M.) of FUDR by mixing about 4×10^8 cells per ml. of each strain in a M 63-aspartate medium, pH 6.3 (PARDEE, JACOB and MONOD 1959). Timed aliquots were taken for the assay of β-galactosidase activity and for plating on selective agar to detect recombinants. In this Hfr H strain the *thr* and *leu* genes are closer than the *lac* (*z*, *i*.) gene to the extremity of the chromosome O; the thr^+ leu^+ Sm^r recombinants were selected in the first place and then the lac^+ recombinants among them were enumerated by making replicas on EMS-lactose-streptomycin agar.

* We dedicate this paper to Prof. L. C. DUNN on the occasion of his retirement.

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*** Public Health Service Training Fellow.

Upon mating, as shown in Fig. 1, β-galactosidase was synthesized at about same rate in the presence as in the absence of FUDR. In the presence of FUDR the zygotes which received genetic determinants were probably unable to complete the process of recombination in the mating medium, because, for it to occur, the replication of genetic material (DNA synthesis) is apparently necessary. The fact that the enzyme was synthesized in this condition confirms the concept of the independence of the expression of genetic information from recombination. Furthermore, it is clear that the *synthesis* of DNA is not required for the phenotypic expression of newly introduced genetic material, a fact which restricts its role in protein synthesis. This result shows a good agreement with the previous findings made in *E. coli* 15 t^-; during thymine starvation, when no DNA synthesis was taking place, induced enzyme synthesis such as that of xylose isomerase (COHEN and BARNER 1955) and β-galactosidase (SPIEGELMAN 1955) has been observed. Finally, the necessity for the existence of DNA, not of its synthesis, for protein synthesis is further emphasized.

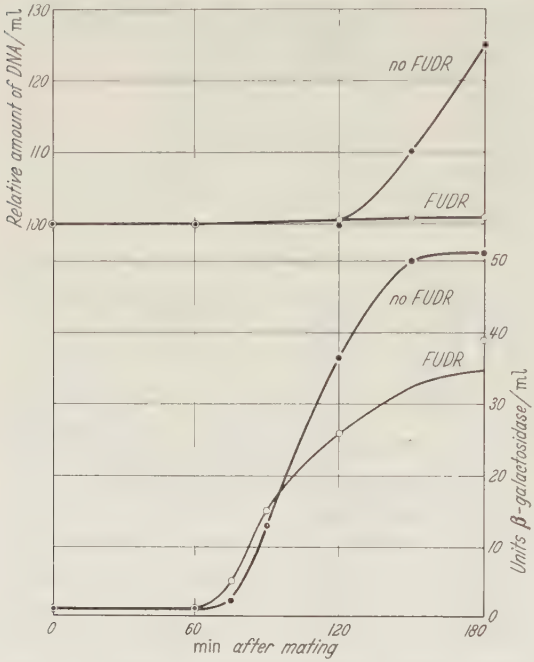

Fig. 1. Synthesis of β-galactosidase by zygotes in the presence and absence of *FUDR*. The mating pairs of *E. coli* K 12, Hfr (H) z^+ i^+ and F^-(W1177) z^- i^+, were mixed at time 0 in M 63-aspartate medium (enriched by 3 mg. of Casamino acids, Difco, per ml.) with threonine, leucine, and vitamin B_1 with and without *FUDR* (1×10^{-4} M.). At 45 min. streptomycin (400 μg. per ml.) was added to prevent the Hfr from making β-galactosidase. At 50 min. Duponol (sodium dodecylsulfate, 30 μg. per ml.) was added to prevent further mating and the cultures were vigorously shaken to separate the mating bacteria. At 60 min. the inducer, methyl-β-D-thiogalactoside (TMG, 5×10^{-4} M.), was added. Timed aliquots were taken for β-galactosidase assay and DNA estimation. One unit of β-galactosidase liberates 1 mμmole 0-nitrophenol from 0-nitrophenol-β-D-galactoside per min. at 28° C, pH 7.0. The amount of DNA was estimated by the diphenylamine reaction. Timed aliquots were treated with cold 5 per cent trichloracetic acid and the precipitates were extracted with hot 5 per cent trichloracetic acid

Noteworthy here is the fact that although the synthesis of DNA, and therefore the recombination process, was prevented in the mating medium in the presence of FUDR, recombinants (*thr*$^+$ *leu*$^+$ *lac*$^+$ *Sm*^{r}) were found on selective agar in its absence. The frequency of such recombinants per initial F^- cell was: with FUDR, 1.4×10^{-5}; without FUDR, 9.4×10^{-6}, when the aliquots from mating medium were plated after 180 minutes of mating. If this discrepancy is significant, it may be explained by the fact that the FUDR-inhibited zygotes resumed DNA synthesis after being plated on the selective plates since it has been shown that thymine-starved cells retain their ability to synthesize more DNA when thymine is returned to them (NAKADA 1960).

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ENHANCEMENT OF ULTRAVIOLET-INDUCED MUTATION IN BACTERIA BY CAFFEINE* **

By

Margaret Lieb

With 4 Figures in the Text

(Received July 24, 1961)

Growth in the presence of caffeine or theophylline has been shown to increase mutation in *Escherichia coli* (Novick and Szilard 1951, Greer 1958). Witkin (1958) reported that the mutagenic effect of ultraviolet light (UV) in *E. coli* was greatly enhanced when irradiated bacteria were grown on a medium containing caffeine. The frequency of tryptophane-independent (try^-) mutations in B/r try^- could be increased up to 100-fold over that obtained by plating on a medium without caffeine. The same treatment produced no detectable increase in mutant frequency in unirradiated bacteria. Doudney and Haas (1959a) using a lower concentration of caffeine than Witkin, observed no effect on mutation in UV-irradiated bacteria.

To account for its effect on mutation after UV, Witkin suggested that caffeine prolonged the post irradiation sensitive period during which mutation can be increased by protein synthesis. However, the current concept of mutation as a copy error during DNA synthesis does not provide any *direct* role for protein synthesis in mutagenesis. It seemed of interest, therefore, to investigate further the effects of caffeine on mutation in irradiated bacteria.

In an attempt to facilitate the comparison of these experiments with those of other workers, we propose that UV-induced microbial mutants be classified as follows:

1. Potential mutants. Organisms that can, under suitable conditions, undergo a stable (genetic) change and graduate to class 2 and 3.

2. Unexpressed stable mutants. Organisms that have undergone a stable change, but do not yet show a mutant phenotype.

3. Expressed mutants. Organisms that have undergone a stable change and show the mutant phenotype.

A stable change is defined as an essentially irreversible event that produces a heritable modification of the phenotype.

This classification is based on the fact that the frequency of mutants recovered after UV treatment is dependent on the environment following irradiation. When irradiated try^- cultures are plated on a medium containing supplements that result in a short latent period followed by several divisions of the try^- cells, a certain number of try^+ colonies eventually appear. Each of these colonies

* Dedicated to Professor L. C. Dunn.

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is presumably the result of a single mutation, so that we may use the terms *mutant* and *mutation* interchangeably. If, however, the irradiated bacteria are first exposed to chloramphenicol for an hour, and then plated on supplemented medium, fewer mutants appear (Witkin 1956). These could be considered stable mutants, and various "challenges" have been used to study the cellular activities associated with attainment of stability or "mutation fixation" (Doudney and Haas 1958). Mutation fixation as determined by "chloramphenicol challenge" occurs more rapidly than fixation (or stability) as determined by incubation in saline, so that the "irreversibility" of the event that results in a mutant is a function of the test. This has led to the correlation of mutation fixation with RNA synthesis (Doudney and Haas 1959b, 1960a) and DNA synthesis (Lieb 1960). Loss of photoreactivability is correlated with DNA doubling in irradiated cells (Doudney and Haas 1960a).

There is less ambiguity, fortunately, in the definition of *expressed mutants*, which are assayed on an unsupplemented minimal medium when mutation to prototrophy is studied, or by the addition of phage or antibacterial agents for the determination of resistant mutants. Expression of UV-induced mutations is preceded by at least a doubling of the DNA/bacterium (Lieb 1959, Haas and Doudney 1959a).

At any time after UV irradiation, the *total* number of mutants that appear on a properly supplemented medium obviously includes those arising from all three classes. The experiments reported here indicate that most of the potential mutants induced by UV are usually lost even in the absence of any deliberate "challenge".

Materials and Methods

A culture of *Escherichia coli* B/r *try*$^-$ (WP 2) was kindly supplied by Dr. E. Witkin. A stock broth culture was inoculated into Difco nutrient broth containing 0.5% NaCl and grown at 37° on a reciprocating shaker. Stationary phase cultures were harvested 3—6 hours after reaching maximum turbidity, and cultures in the logarithmic phase were harvested when the cell concentration was $3—5 \times 10^8$ ml. In some experiments, the method of Doudney and Haas (1958, 1959b) was used to obtain early log phase cultures in amino-acid supplemented minimal medium. Cultures were chilled, washed once with cold 0.9% NaCl, and suspended in cold saline to give $1—2 \times 10^9$ bacteria/ml.

The organisms were irradiated in a large chilled dish, and agitated constantly during treatment. The UV source was 2 Westinghouse Sterilamps mounted side by side, delivering approximately 8 ergs/mm^2/sec at a distance 97 cm from the source, as calibrated with phage T 2 survival curves. In a typical experiment, 6 ml aliquots of irradiated bacteria were diluted into flasks containing 60 ml prewarmed nutrient broth plus appropriate concentrations of the substances to be tested. One flask was immediately chilled, the bacteria were centrifuged and washed 1 time with cold saline, and resuspended in 6 ml cold saline. This sample was used as a control in later biochemical measurements, and also to determine the per cent survival. The remaining flasks were incubated on the shaker in the dark at 37° and the contents were washed and reconcentrated as in the control. If further treatment was desired, the bacteria were reinoculated into additional flasks, and the procedure repeated.

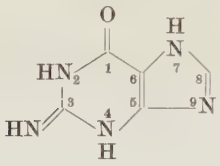
The reconcentrated suspensions of bacteria were assayed for viable count using 0.9% saline as the diluting medium. A modified Gray and Tatum's minimal medium (Ryan and Schneider 1959) containing 1.2% Bacto agar and 0.2% glucose but no asparagine was used for plating. *Try*$^-$ bacteria and also total mutants were assayed on minimal medium containing 5% (by volume) nutrient broth. This is equivalent to the SEM medium of Witkin (1956). Bacteria were distributed on the plates by spreading at room temperature, and the plates were then rapidly brought to 37° in an incubator. *Try*$^-$ assay plates were routinely counted after 48 hours, and plates containing *try*$^+$ mutant colonies were counted at 72 hours.

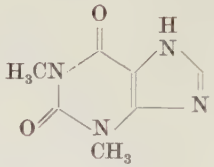
Four ml samples of reconcentrated bacteria were extracted once with cold 0.25 N perchloric acid, followed by extraction of the nucleic acids for 25 min at 80° with 0.5 N PCA. RNA was determined using the orcinol reaction (BROWN 1946) and the method of BURTON (1956) was used to assay DNA. Aliquots of washed bacteria were used without PCA extraction for determination of protein (LOWRY et al., 1951). Purines, purine analogues, RNA and DNA (salmon sperm) were obtained from the California Corporation for Biochemical Research. Chloramphenicol was the gift of Parke, Davis, Inc.

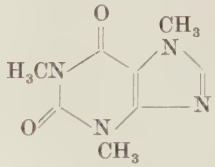
Results

Effect of caffeine on synthetic activities of E. coli B/r

Caffeine and theophylline are analogues of guanine (Schema) and might be expected to affect both RNA and DNA synthesis. To compare the effect of


Guanine


Theophylline


Caffeine

caffeine on irradiated and unirradiated cells, unirradiated and UV-irradiated aliquots of the same culture were incubated with caffeine for an hour. Table 1 shows that caffeine reduced slightly the amount of RNA and protein synthesized during 70 min incubation by both control and irradiated bacteria. However, there was a greater reduction in the amount of DNA synthesized, particularly

Table 1

	Treatment	Protein		RNA		DNA	
		rel. amt.	rel. increase	rel. amt.	rel. increase	rel. amt.	rel. increase
A	0	100	—	100	—	100	—
	70 min NB	297	197	450	350	545	445
	70 min NB + Cf	270	170	385	285	458	358
	$\frac{NB+Cf}{NB}$		0.86		0.85		0.80
AUV	UV	100	—	100	—	100	—
	70 min NB	297	197	420	320	485	385
	70 min NB + Cf	265	165	365	265	334	234
	$\frac{NB+Cf}{NB}$		0.84		0.83		0.61
BUV	UV	100	—	100	—	100	—
	70 min NB	340	240	470	370	294	194
	70 min NB + Cf	280	180	385	285	202	102
	$\frac{NB+Cf}{NB}$		0.82		0.82		0.53

Culture. WP 2 was in the logarithmic phase in NB at 37°. Caffeine was added to aliquot A to give a concentration of 0.1%, and the culture was harvested 50 min later. No caffeine was added to aliquot B.

UV. 80 ergs/mm².

Treatment. After UV, cells were diluted 6:60 in NB or NB + 0.05% caffeine and grown at 37° for 70 min.

in the irradiated sample. Similar results were obtained using theophylline and stationary-phase bacteria. In addition, the data in Table 1 show that incubation in caffeine before UV irradiation did not make bacteria less sensitive to the effects of caffeine added later.

While chloramphenicol prevents DNA synthesis when added immediately after UV irradiation (Harold and Ziporin 1958), caffeine does not prevent DNA synthesis in the irradiated bacteria or delay its onset, but reduces the rate of synthesis. This is shown by experiments in which irradiated bacteria were inoculated into broth or broth plus caffeine and assayed after incubation for

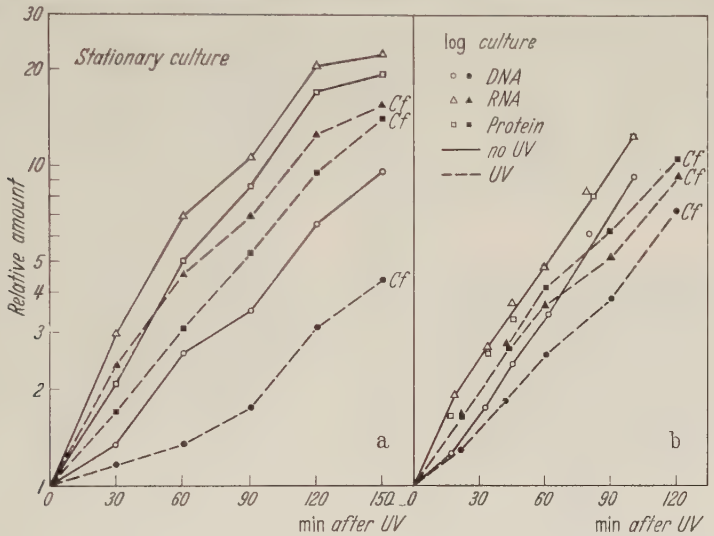


Fig. 1a and b. a A stationary phase culture of WP 2 was exposed to 120 ergs UV/mm². Aliquots of 3 or 6 ml were inoculated into flasks containing 60 ml NB + 0.1 % caffeine and incubated on a shaker at 37°. Flasks were chilled at the indicated times, and the cells reconcentrated in the cold. Leveling — off of RNA and protein after a 10 fold increase is attributable to suboptimum growth conditions in the flasks at high cell concentrations. b A logarithmic phase culture of WP 2 was given 80 ergs UV/mm². Aliquots were inoculated into flasks containing 60 ml NB 0.05 % caffeine, and handled as in 1a

various times. In a stationary phase culture (Fig. 1a) the rates of RNA and protein synthesis, and particularly DNA synthesis, were reduced during the first 60 min of incubation in caffeine. After this time, these rates of synthesis appeared to approach those of the control. Fig. 1b records the results of a similar experiment using logarithmic phase cells and a lower concentration of caffeine. In this experiment, caffeine had little effect on protein and RNA synthesis during the first 40—60 min, although DNA synthesis was slowed. After 60 min, the rates of protein and RNA synthesis decreased so that RNA, DNA and protein increased at approximately the same rate. These results and those of additional experiments indicate that the major effect of caffeine on the overall synthetic activities of irradiated bacteria is to reduce, temporarily, the rate of DNA synthesis.

Optimal caffeine concentration

Witkin (1958) reported that as the concentration of caffeine in the medium increased, the length of the post-UV lag in bacterial division also increased, and

this increased lag presumably resulted in a longer sensitive period. We have investigated the effect on DNA, RNA and protein synthesis of exposing UV *try*-bacteria to various concentrations of caffeine for 90 min. Fig. 2 shows that the effectiveness of any concentration of caffeine in increasing the mutation frequency is not proportional to its ability to slow the synthetic activities of the culture. There was no obvious relationship between the relative amounts of DNA and protein synthesized during exposure to caffeine and the level of mutation frequency obtained.

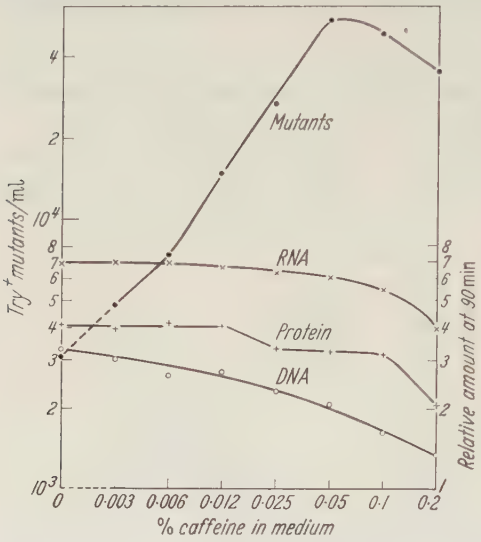


Fig. 2. *Culture:* WP 2 stationary culture. *UV:* 120 ergs/mm². *Treatment:* Seven ml of irradiated cells were inoculated into 50 ml NB containing caffeine as indicated. After 90 min at 37°, each culture was reconcentrated to 7 ml. 1.00 = Amount of DNA, RNA and protein immediately after UV

Effect of varying the exposure time

Fig. 3a and b show the effect of growing irradiated bacteria in caffeine for varying lengths of time. The maximum mutant concentration was observed after about 60 min incubation for log-phase cells, and 90 min for cells in the stationary

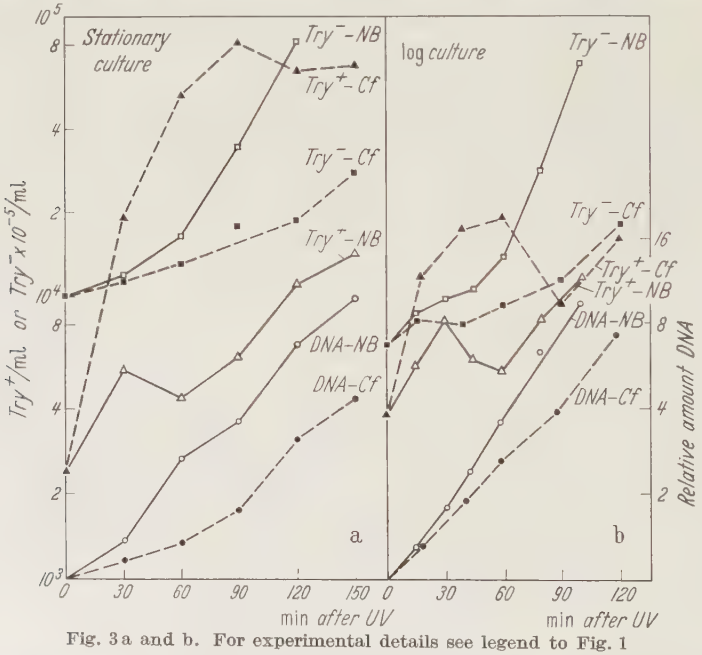


Fig. 3a and b. For experimental details see legend to Fig. 1

phase before irradiation. In each case, the amount of DNA had approximately doubled at the time that further exposure to caffeine produced no additional

mutants. The significance of the drop in mutant concentration observed with longer incubation is not yet clear; however, the number of *try*$^+$ mutants eventually increased due to cell division.

The initial increase in mutant concentration after incubation in broth without caffeine can be attributed to the effect of amino acids on UV-induced mutation in broth-grown bacteria which was reported by WITKIN in 1956: the number of mutants that could be recovered from a culture that had been grown in an amino acid-rich medium was influenced by the concentration of amino acids in the medium on which the cells were grown immediately after UV. We have repeatedly observed that aliquots of irradiated broth-grown bacteria, particularly bacteria in the log phase, give rise to more mutants when incubated in "100%" NB for 30 min before plating than when plated immediately on 5% NB medium. This effect has been termed "mutation stabilization" by DOUDNEY and HAAS (1958) but in the absence of evidence that amino acids (or other broth constitutents) do, indeed, stabilize mutations this term appears unwarranted. WITKIN (1956) showed that broth in the post-irradiation medium decreased the length of the lag phase and increased the growth rate of irradiated bacteria, and this effect was directly correlated with an increase in mutation. It should be noted that a post-UV treatment that *decreases* the length of the lag period, e.g. growth in broth, can increase the number of mutants recovered, whereas a treatment that *increases* the lag, e.g., the addition of caffeine, has the same effect. Thus, we have additional evidence that the effect of caffeine on mutation frequency is not directly related to its effect on the growth rate of irradiated cells.

Table 2

Time of caffeine addition	Time of mutant assay min	*Try*$^-$/ml ($\times 10^{-7}$)	*Try*$^+$/ml	*Try*$^+$/10^7 *Try*$^-$
—	30[1]	143	2512	17.6
0	60	141	35000	249
0	90	144	57600	398
0 + 60 min	90	175	50700	290
0	120	164	51000	310
0 + 60 min	120	168	44300	265
0	180	518	104800	202
0 + 90 min	180	242	60800	251

[1] Exposure time giving highest mutant frequency in NB.

Culture. WP 2 stationary phase.

UV. 120 ergs/mm^2.

Treatment. 6 ml aliquots of irradiated cells were inoculated into 59 ml NB. Caffeine to give a final concentration of 0.1% was added before inoculation (0 time). At a later time, a second caffeine addition was made to some tubes, giving a final concentration of 0.2%.

Possible inactivation of caffeine by bacteria

The loss of the effectiveness of caffeine after a certain time led to a consideration of the possibility that caffeine was inactivated by the cells. In order to test this notion, additional caffeine was added to cultures at the time that caffeine seemed to be losing its effect. As shown in Table 2, the addition of more caffeine at 50 or 90 min resulted in a slight decrease in the number of mutants per ml of irradiated culture. It appears more likely that caffeine loses its effectiveness due to a change in a cellular component sensitive to it rather than due to a drop in the concentration of the analogue.

Time of caffeine addition

As shown in Fig. 4, caffeine added after the first 20 min of growth in broth had no effect on mutation; at this time, the DNA had increased only 15%. Over 50% of the total recoverable mutants were lost when the irradiated cells were incubated in broth for only 5 min before caffeine addition. During this time there was no measurable synthesis of DNA and protein, and only a 25% increase in RNA. The decline in the ability of caffeine to increase the mutation frequency can be regarded as a loss of potential UV-induced mutants, mutants which are protected by caffeine if it is added in time. The loss of these potential mutants is not correlated in any obvious way with synthesis of any cell component measured, and is very similar to the "mutation frequency decline" that occurs when irradiated cells are put under sub-optimal growth conditions.

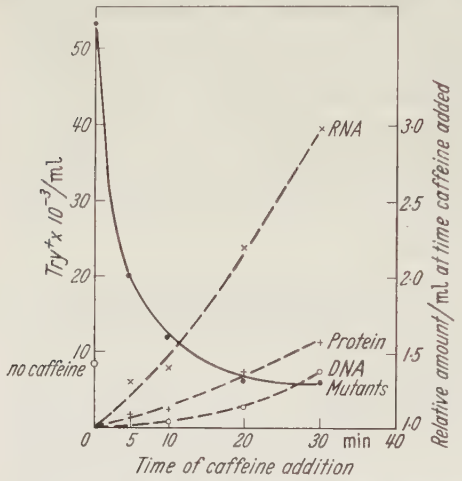


Fig. 4. *Culture:* Stationary phase. *UV:* 200 ergs/mm² (100% survival). *Treatment:* At t = 0, a series of flasks containing 34 ml NB were each inoculated with 6 ml irradiated bacteria. Immediately thereafter, and after 5, 10, 20 and 30 min at 37°, one flask was chilled, and caffeine was added to a second flask to give a final concentration of 0.2%. After caffeine addition, cultures were incubated an additional 60 min. All cultures were reconcentrated to 6ml

Effect of caffeine in the presence of Chloramphenicol

Incubation of irradiated *try*⁻ bacteria in the presence of chloramphenicol (CMP) can result in the loss of 80% or more of total mutants recoverable on SEM medium (WITKIN 1956). There have been conflicting interpretations of the action of chloramphenicol: LIEB (1960) presented evidence indicating that CMP delayed DNA synthesis, thus allowing additional time for a cellular repair mechanism to remove an unspecified "mutagen", and DOUDNEY and HAAS (1960b) countered with data to support their view that CMP "promotes *active* mutation frequency decline".

WITKIN (1958) showed that caffeine delayed the loss of mutants that occurred when irradiated *try*⁻ bacteria were placed in minimal medium. The data presented in the previous section suggested that caffeine interfered with a process that reduces the number of potential mutants in bacteria even when they are placed under optimum growth conditions after UV irradiation. If the same process were responsible for the loss of mutations caused by chloramphenicol, one would expect caffeine to interfere with CMP-induced "mutation frequency decline".

A comparison of lines 1, 7 and 10 of Table 3 shows that CMP was less effective in promoting the decline in mutant frequency when caffeine was also present in the medium. However, 30 min incubation in caffeine alone (line 4) resulted in the recovery of many more mutants than incubation in caffeine plus CMP (line 10). One way to account for this apparent low protective action of caffeine in the presence of chloramphenicol, and retain the notion that both drugs influence

Table 3

No.	Incubation medium		Amount relative to 0 time			*Try*⁻/ml ($\times 10^{-7}$)	*Try*⁺/ml	*Try*⁺/10⁷ *Try*⁻
	0—30 min	30—60 min	DNA	RNA	Protein			
1.	NB	—	110	240	144	235	2500	10.6
2.	NB	NB	340	1220	450	322	4500	14.0
3.	NB	NB+CF	198	570	360	287	4492	15.6
4.	NB+CF	—	104	184	131	166	23500	141
5.	NB+CF	NB	237	595	350	290	47000	159
6.	NB+CF	NB+CF[1]	270	675	490	317	87160	275
7.	NB+CMP	—	97	141	97	198	480	2.4
8.	NB+CMP	NB[1]	195	630	310	400	1050	2.6
9.	NB+CMP	NB+CF[1]	141	432	227	202	1540	7.7
10.	NB+CF+CMP	—	75	161	104	213	1095	5.1
11.	NB+CF+CMP	NB[1]	266	715	360	315	1620	5.1
12.	NB+CF+CMP	NB+CF[1]	160	500	267	223	37200	166

[1] These cultures incubated 30 additional minutes in the indicated medium.

Culture. WP 2 stationary culture.

UV. 200 ergs/mm².

Treatment. 13 ml lots of irradiated bacteria were diluted into 130 ml NB containing 0.05% caffeine and/or 15 μg CMP as indicated (lines 1, 4, 7, 10). After 30 min at 37°, the cultures were chilled, washed, and resuspended to 13 ml. Three ml samples were inoculated into 60 ml broth±0.05% caffeine. After 60—90 additional minutes at 37°, the bacteria were again washed and reconcentrated.

the same process, is to postulate that although potential mutants are protected while caffeine is present, they become susceptible to the decline process as soon as caffeine is removed. By incubating in caffeine after the removal of CMP, one should be able to prevent this decline and recover a larger frequency of mutants. A comparison of lines 6 and 12 shows that over 60% of the total recoverable mutants (as determined by incubation in caffeine for 120 min) survived the 30 min in CMP plus caffeine. On the other hand, 90% of the potential mutants were lost after a 30 min period of incubation in broth (line 3 *vs* line 12).

Although the data presented here do not exclude the possibility that CMP accelerates a normally-present decline process, they clearly indicate that in the case of potential mutants protected by caffeine, CMP promotes a loss of mutants that occurs *after its removal*. This effect of CMP treatment is correlated with a delay in DNA synthesis when cells are subsequently put into broth (compare lines 2, 5, 8, 9).

Caffeine treatment followed by chloramphenicol "challenge"

If the effect of caffeine were due to its direct participation in the stabilizing event that produces mutants after UV, the mutants that appear after growth in caffeine should be resistant to a "challenge" such as incubation in chloramphenicol. Table 4 shows that incubation in caffeine for 30 min results in a significant increase in mutation frequency after UV. However, a large fraction of these mutants do not appear if the culture is subsequently incubated in chloramphenicol. The experiment of Table 3 showed that potential mutants present as a result of incubation in caffeine plus CMP were lost during subsequent

incubation in broth. In effect, caffeine increases the number of *potential mutants* remaining at a given time after UV, and need not be present during the actual stabilizing event in order to increase the mutation frequency.

Incubation in caffeine before UV

Exposure of cultures to caffeine for periods of 10—70 min before UV did not result in an increase in the frequency of mutants recovered either with or without subsequent growth in caffeine. A suboptimal concentration of caffeine was used for the post treatment in order to permit the detection of even small changes in the effectiveness of the analogue. Table 1 gives data showing that growth in caffeine did not change the sensitivity of the synthetic activities of a culture to caffeine added after irradiation.

Irradiation of caffeine

No change in the activity of caffeine was detected after doses up to ten times as high as any given the bacteria in these experiments. Irradiation of bacteria

Table 4

Expt. No.	Treatments after UV		*Try*⁻/ml ($\times 10^{-7}$)	*Try*⁺/ml	*Try*⁺/10⁷ *Try*⁻
	0—30 min	30—60 min			
113	NB	—	290	25000	86
	NB+Cf	—	270	59000	220
	NB+Cf	NB+CF+CMP	231	16000	70
	NB+Cf	NB+CF	271	131400	485
	0—30 min	30—90 min			
395	NB	—	182	5400	30
	NB+CF	—	179	35700	200
	NB+CF	NB+CMP	134	12960	97
	NB+Cf	NB+Cf	213	95000	445
405	NB	—	205	4800	24
	NB+CF	—	185	31500	171
	NB+Cf	NB+CMP	149	16600	110
	NB+CF	NB+CF	172	91000	530

Cf=Caffeine; CMP=Chloramphenicol 20 ug/ml.

Expt. 113: Stationary phase WP 2 was exposed to 300 ergs/mm² UV giving 78% survival. Six ml aliquots of irradiated cells were inoculated into 44 ml NB or NB+2 mg/ml caffeine. At 30 min incubation, CMP was added to one of the NB+CF flasks and another NB+CF culture was chilled. After 30 additional minutes at 37°, the remaining flasks were chilled. All cultures were washed and reconcentrated in saline.

Expts. 395 and 405: Stationary phase WP 2 was given 200 ergs/mm² UV allowing 100% survival. Thirteen ml aliquots of the cells were inoculated into broth with and without 1 mg/ml caffeine. After 30 min at 37°, the cultures were washed and reconcentrated, and a portion of each suspension reinoculated into fresh medium containing the supplements listed. After incubation for 60 min, the bacteria were washed and reconcentrated.

in the presence of caffeine induced few if any mutations due to the adsorption of UV by the analogue. The concentration of caffeine in these experiments was 0.1%.

Effect of normal bases and ribosides

Novick and Szilard (1952) and Novick (1956) reported that adenosine and guanosine, but not the free bases, antagonized the mutagenic effect of theophylline and caffeine. As little as 5 mg/l guanosine completely overcame the increase in the mutation rate to T 5 resistance due to the presence of 150 mg/l theophylline. In repeated attempts, we have been unable to reduce the mutagenic affect of caffeine or theophylline on UV bacteria by the addition of any of the normal bases or their ribosides. Results of a typical experiment are presented in Table 5. Additional experiments in which the molar ratio of caffeine:guanosine was as high as 1:3 gave similar results. Shankel (personal communication) has obtained independent evidence that normal purines and pyrimides do not alter the effect of caffeine after UV.

Table 5

Addition	*Try* /ml ($\times 10^{-7}$)	Try^+/ml	$Try^+/10^7$ Try^-
None.	260	5670	22
0.1% Adenosine	319	8240	26
0.1% Guanosine	223	8000	36
0.2% Caffeine	163	28760	176
0.2% Caffeine + 0.1% Adenosine	220	39000	177
0.2% Caffeine + 0.1% Guanosine	184	32000	174

Culture. WP 2 grown in M medium by the method of Doudney and Haas.

UV. 80 ergs/mm², giving 80% survival.

Treatment. Twelve ml irradiated cells (8×10^8/ml) inoculated into aliquots of 48 ml M medium + 0.2% casein hydrolysate, 200 μg tryptophane/ml, and caffeine and/or purine ribosides as listed above. After incubation at 37° for 60 min, each culture was washed and resuspended to 6 ml. (0.2% caffeine =0.01 M, 0.1% guanosine or adenosine=0.003 M).

Discussion

Koch (1956a) and Koch and Lamont (1956) reported that caffeine and related methyl purines inhibited purine nucleoside phosphorylases, transribosidases and hydrolases in *E. coli* extracts. They suggested that the mutagenicity of certain base analogues could result from a lowering of the concentration of normal purines and purine derivatives which would tend to increase the probability of an error in DNA replication. The fact that normal purine ribosides antagonize the mutagenicity of caffeine and theophylline (Novick and Szilard 1952) supports this hypothesis. However, added purine ribosides did not modify the effect of caffeine present in the growth medium of UV-irradiated bacteria. Moreover, benzimidazole, which was reported to be mutagenic for unirradiated cells (Novick 1956, Greer 1958) significantly reduced the frequency of mutation after UV (Witkin 1958, Lieb, unpublished). If we restrict comparisons to strain WP 2, we find that benzimidazole, but not caffeine or theophylline, is markedly mutagenic for unirradiated bacteria, while the reverse is true for irradiated cells. Thus, it appears likely that the pathways by which caffeine and theophylline produce mutations differ in irradiated and unirradiated bacteria.

In considering the ways in which base analogues could influence the recovery of mutants after UV, we would like to make two basic assumptions:

(a) UV produces photoproducts (PP) whose presence during DNA synthesis results in copy errors. Known photoproducts that could be formed in nucleic acid include the hydration products of cytosine and uracil (Sinsheimer 1954,

1957) and a dimer of thymine (BEUKERS and BERENDS 1960; WACKER, DELLWEG and WEINBAUM 1960).

(b) The irradiated bacterium has available a "dark recovery" (DR) system, probably enzymatic, which removes or alters PP so as to reduce the probability of mutation at DNA synthesis.

Evidence for the enzymatic nature of mutation frequency decline in the dark has been summarized by DOUDNEY and HAAS (1959b). The data presented here indicate that caffeine inhibits mutation frequency decline in the presence of chloramphenicol and during the lag in DNA synthesis following CMP treatment. Additional studies indicate that caffeine also increases UV killing of nonlysogenic bacteria, and induction of phage development in a lysogenic strain (LIEB 1961).

As mentioned in the introduction, the nature of the stabilizing event is still controversial. For the purposes of this discussion, the term "stabilizing event" can be substituted for "DNA synthesis". The relationship between the stabilization of potential mutations and DNA synthesis has not yet been studied systematically after caffeine treatment. However, it has been shown that a short period of incubation in caffeine, during which little or no DNA is made, increases the frequency of mutants that can be recovered, and that the majority of these mutants are not stable since they can be lost during "chloramphenicol challenge" or division delay. Studies of the relationship between DNA synthesis and the fraction of total mutants which fall into the class of stable expressed mutants revealed no difference between samples of a culture growing in broth and samples growing in broth plus caffeine. Thus, there is every indication that in these experiments caffeine merely enhances the effect of UV, and has no specific mutagenic action of its own.

Some possible mechanisms of caffeine action may now be considered. Hypothesis 1: Caffeine is incorporated into DNA when DNA containing PP is used as a template.

Previous studies have failed to show incorporation of caffeine into the DNA of normal *E. coli* (KOCH 1956b, GREER 1958). However, we should consider the possibility that UV induces the formation of a demethylating enzyme that would remove the methyl group in the 7 position and permit the formation of a stable caffeine deoxyriboside. In this case, one would still expect guanosine and adenosine to compete with caffeine, but they were found to be ineffective. Moreover, theophylline, which is unsubstituted in position 7, is no more effective than caffeine or theobromine in promoting mutation in UV treated bacteria. The incorporation hypothesis also fails to account for the fact that in several experiments, exposure to caffeine caused an increase in mutation frequency even though no DNA was made during the caffeine treatment.

Hypothesis 2: Caffeine combines with DNA and/or RNA and prevents the access of DR enzyme to PP. Acriflavine, which combines with DNA and RNA, has an effect similar to that of caffeine on mutation after UV (WITKIN 1961). WITKIN has suggested that acriflavine and other dyes prevent the repair of UV-induced changes in DNA. If DNA is added to broth containing a suitable concentration of acriflavine, some of the dye is removed, lowering the mutagenicity of the medium for irradiated bacteria. However, mixing DNA or irradiated DNA (up to 1 mg/ml) with 0.5 mg/ml caffeine resulted in no loss of

caffeine activity. The optimum concentration of caffeine for post-UV mutagenicity is about 100 times the optimum acriflavine concentration, so that if there were a weak reversible attachment of caffeine to DNA it would be difficult to demonstrate in this type of experiment. Studies with radioactive caffeine are projected.

Hypothesis 3: Caffeine competes with PP for DR enzyme. None of the photoproducts mentioned above has a marked resemblance to a methylpurine, although the thymine dimer also contains methyl groups. In a preliminary study, thymine dimer was prepared by irradiating thymine solutions (Beukers and Berends 1960) and added to mixtures of caffeine, broth and irradiated bacteria. No effect of the dimer was observed. Studies of substances related to caffeine are continuing in an attempt to determine the part of the molecule responsible for its effect on mutation after UV.

Hypothesis 4: Caffeine suppresses the formation or release of DR enzyme. In this case, one would expect that the pre-UV incubation in caffeine would increase the mutation frequency observed without caffeine, or with suboptimal amounts of caffeine in the post-UV medium. The experiments reported here, and those of Shankel (1961) do not support this hypothesis. On the other hand, such negative results are compatible with the notion that the DR system is induced by irradiation.

We must conclude, then, that while the effects of caffeine can be accounted for by the general notion that certain methylpurines interfere with the removal of a mutagenic product of UV irradiation, we have no direct evidence to support a more precise hypothesis.

Although there have been numerous studies suggesting that pre-lethal damage is repaired in irradiated cells in the dark under various conditions, Kimball et al (1959) recently proposed that irradiated cells have a built-in system for repairing pre-mutational damage in the absence of photoreactivation. In *Paramecium*, the amount of genetic damage decreased with increase in time between irradiation and DNA synthesis. Although there are a few apparent exceptions, most post-UV effects on bacterial mutation can be understood in the light of this general idea. In the case of mutation from try^- to try^+, one can assume that a certain amount of photoproduct is present in the DNA and/or RNA. An enzyme (or, perhaps, more than one) immediately starts to remove or breakdown PP; meanwhile, other reactions result in a resumption of DNA synthesis. Once DNA synthesis starts, the probability of a copy error/DNA strand will be proportional to the amount of PP present at the time of its replication. The effectiveness of a substance as an "enhancer" of UV-induced mutation would depend on its relative effectiveness as an inhibitor of the repair mechanism and as an inhibitor of DNA synthesis. Any treatment delaying DNA synthesis while allowing DR activity would decrease the probability of mutation and, if the same PP's are involved, increase the probability of survival. One would also expect that some substances would enhance the activity of the DR system rather than increase the time available for its effective action.

If caffeine is present in the culture medium, it is possible to recover over 10 times as many mutants as would be recovered if the cells were simply incubated in broth. One must, therefore, conclude that B/r WP 2 bacteria normally repair over 90% of premutational damage produced by low doses of UV.

Summary

The addition of caffeine or theophylline to the growth medium of irradiated *E. coli* B/r *try*⁻ resulted in a 10-fold or greater increase in the frequency of *try*⁺ mutants. These observations extend those of WITKIN (1958). Caffeine produced a slight reduction in the rate of RNA and protein synthesis, and a somewhat greater but temporary reduction in the rate of DNA synthesis. The analogue must be added immediately after UV-irradiation to produce its optimal effect, and the ability of an irradiated culture to respond to caffeine was lost completely after 20 min incubation in broth. Normal purine ribosides did not compete with caffeine. The optimal exposure time to caffeine was correlated with the time of DNA doubling, but marked increases of mutation frequency resulted when caffeine was present for 30 min in the absence of DNA synthesis. Incubation in caffeine before irradiation had no effect. Caffeine also reduced mutation frequency decline caused by incubation of irradiated bacteria in chloramphenicol. It is suggested that caffeine interfers with a "dark repair" enzyme system which removes a UV photoproduct (s) whose presence during DNA synthesis leads to mutation.

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GENETIC ANALYSIS OF STREPTOMYCIN-RESISTANCE AND -DEPENDENCE IN *CHLAMYDOMONAS* * **

By

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With 1 Figure in the Text

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The literature of genetics has included reports of non-chromosomal inheritance beginning with the classical studies of C. E. Correns (1937). As yet, however, no unified concept has emerged of the role of these factors in cell heredity. In this paper, experiments are described in which the alga, *Chlamydomonas*, is shown to carry non-chromosomal determinants of streptomycin-resistance and -dependence. The results demonstrate that both chromosomal and non-chromosomal determinants can individually alter the response of cells to streptomycin, and, furthermore, that these factors can interact at the biochemical level without altering their independent patterns of inheritance. These findings will be discussed in relation to current hypotheses concerning the nature of non-chromosomal determinants.

A. Material and Methods

All strains of *Chlamydomonas* used in these studies were derived from the wild-type strain, C 137, isolated as a zygote by G. M. Smith, and germinated to provide a pair of strains of the two mating types. Two other pairs of strains similarly isolated by Smith from other parts of the United States were employed in the special outcrosses described below. Methods of cultivation and of genetic analysis are essentially the same as those previously described (Sager 1955) except that zygotes were dissected by hand for tetrad analysis.

It may be recalled that in this species, mating type is determined by a single gene difference, and that clones of each mating type, *plus* and *minus*, are haploid and may be cultivated indefinitely as vegetative strains. When cells of the two mating types are mixed, pairing occurs leading to fusion and formation of the diploid zygote. After a maturation period of a few days, the zygote (which does not multiply vegetatively) undergoes meiosis, giving rise to four daughter cells, which are the four products of meiosis. The cells may be separated at this time, and each will produce a clone; thus, tetrad analysis is feasible, and in actuality readily performed.

Recrystallized streptomycin used in these studies was the gift of Merck & Co. Chromosomal gene pairs used as markers are: *mt* + / *mt* —, scored as previously described, *act-r*/*act-s*, resistance and sensitivity to 10 μg/ml of actidione (Upjohn Co.); *ms-r*/*ms-s*, resistance and sensitivity to 500 μg of DL-methionine-dl-sulfoximine per ml; *sr*-100/*ss* resistance and sensitivity to 100 μg of streptomycin per ml. The spot test method used for scoring drug resistance consists of inoculating loopfuls of logphase cell suspensions onto agar plates with

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and without added antibiotic, and scoring for growth. Under test conditions, sensitive cells do not grow at all on drug-containing plates. The gene pair *paba* + /*paba* was also readily scored on spot plates with and without added growth factor.

B. Experimental

In previous reports (Sager 1954, 1955) the patterns of inheritance of two streptomycin-resistant strains of *Chlamydomonas reinhardi* were described. One of these strains is resistant to 500 μg of streptomycin per ml, as shown by the ability of every cell to form a viable colony on agar containing 500 μg per ml but not higher concentrations of streptomycin. In crosses with sensitive strains, the resistance segregates in a non-chromosomal manner: resistant strains of mating-type *plus* transmit the property to all progeny without segregation, and resistant strains of mating type *minus* do not transmit the property to any progeny. Thus, the genetic factor responsible for this level of resistance (originally called *sr-2* and now called *sr-500*) exhibits a pattern of inheritance which is both non-chromosomal and uniparental. The other strain is resistant only to 100 μg of streptomycin per ml and the resistance segregates as a chromosomal gene difference. The gene (originally called *sr-1* and now called *sr-100*) is located about 15 map units from its centromere (Sager 1955) in linkage group IX (Sager, unpublished; Levene and Ebersold 1960).

I. Inheritance of sd

Only one streptomycin-dependent strain (mating-type *plus*) has been found in tests of some 10,000 colonies which appeared as mutants on streptomycin-agar plates and were classified for resistance level over the past several years during studies of the induction of resistance (Sager, unpublished). The *sd* strain was detected by poor growth on minimal medium but heavy growth on agar containing 100 μg of streptomycin per ml. The test method, developed for induction studies, consisted of picking new mutant colonies into liquid media without streptomycin for a few divisions to obtain log phase cells for spottesting. It may have resulted in the loss of any mutants so dependent as to require streptomycin in the medium at every division. The *sd* strain described here does not have such a stringent requirement during vegetative growth. Its special requirements during germination will be discussed below.

1. Zygote germination studies. In crosses of *sd* × *ss*, one has the problem of whether to germinate zygotes in media with streptomycin, thereby killing sensitive progeny, or on media without streptomycin, leading perhaps to the loss of dependent progeny. Both methods were tried. Since zygotes are matured on one set of plates, and then transferred to fresh plates for germination, four combinations are possible: maturation on streptomycin-agar, and germination on media with or without streptomycin; maturation on non-streptomycin-agar, followed by germination with or without streptomycin. A comparative test showed that only zygotes that were both matured and germinated on streptomycin-agar gave rise to viable progeny. Zygotes matured on streptomycin agar did germinate in the absence of streptomycin, but the progeny all died. Zygotes matured without streptomycin did not germinate at all.

These results show that the requirement for streptomycin is already expressed in the *sd*/*ss* zygote; *sd* is dominant in the sense that the presence of streptomycin is necessary for germination.

2. Crosses between dependent and sensitive strains. The *sd* strain initially isolated was crossed with a *paba*-strain of the opposite mating type. Germination was about 90% on streptomycin-agar, and of the 70 zygotes observed, 25 were dissected for tetrad analysis. All progeny were uniformly streptomycin-dependent, with no segregation of sensitivity, although the sets segregated normally for the chromosomal markers, mating type and *paba*, as shown in Table 1.

Table 1. *Classification of progeny from the cross: sd mt+ paba+ × ss mt— paba—**

Progeny classes	Number and type of zygote segregation of chromosomal markers
(1) *sd mt+ paba+*; *sd mt— paba—*	1 Parental ditype
(2) *sd mt+ paba—*; *sd mt— paba+*	1 Nonparental ditype
(3) *sd mt+ paba+*; *sd mt+ paba—*; *sd mt— paba+*; *sd mt— paba—*	11 Tetratype
(4) *sd paba+*; *sd paba—* (not tested for *mt*)	12
	25

* All tetrads showed 2:2 segregation for each pair of chromosomal alleles, but no segregation of *sd*/*ss*; all progeny were *sd*.

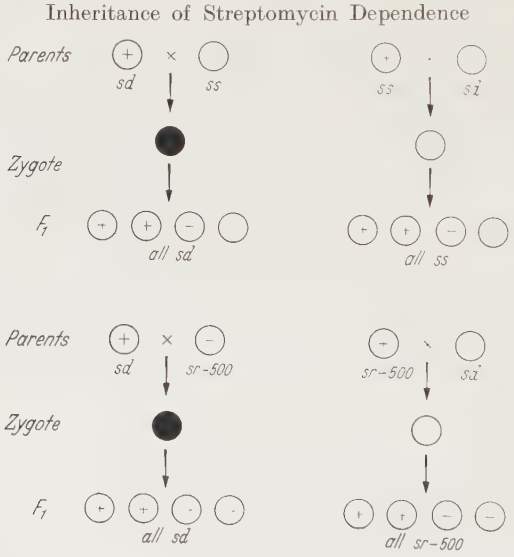

Fig. 1. Streptomycin dependence of progeny depends upon the mating type (shown as + and —) of the *sd* parent. Only *sd mt+* parents transmit *sd* to their progeny; in such crosses the zygotes have an absolute streptomycin dependence. In the reciprocal cross the zygotes lose their dependence before meiosis and progeny are all non-dependent

This pattern of inheritance of streptomycin dependence, with uniform transmission to all progeny, parallels the results previously obtained in crosses of *sr*-500 *mt+* × *ss mt—* (SAGER 1954). The same parallelism was found in backcrosses of the F_1 progeny.

One *sd* F_1 strain of mating type *plus* and one of mating type *minus* were each crossed with a sensitive strain of the opposite mating type. Dependence was transmitted to all progeny when the *sd* factor was carried by the parent of mating type *plus*, and to no progeny when carried by parents of mating type *minus*. The results are shown in Table 2 and Fig. 1.

Table 2. *Classification of progeny from F_1-backcrosses**

Progeny classes	Number and type of zygote segregation of chromosomal markers
Cross 1: *sd mt+ act-r* × *ss mt− act-s*	
(1) *sd mt+ act-r*; *sd mt− act-s*	5 Parental ditype
(2) *sd mt− act-s*; *sd mt− act-r*	3 Nonparental ditype
(3) *sd mt+ act-s*; *sd mt− act-s*; *sd mt+ actr-r*; *sd mt− act-r*	7 Tetratype
(4) *sd act-r*; *sd act-s* (not scored for *mt*)	8
	23
Cross 2: *ss mt+ ms-s* × *sd mt− ms-r*	
(1) *ss mt+ ms-s*; *ss mt− ms-r*	0 Parental ditype
(2) *ss mt+ ms-r*; *ss mt− ms-s*	0 Nonparental ditype
(3) *ss mt+ ms-s*; *ss mt− ms-s*; *ss mt+ ms-r*; *ss mt− ms-r*	6 Tetratype
(4) *ss ms-s*; *ss ms-r* (not scored for *mt*)	13
	20

* All tetrads showed 2:2 segregation of each pair of chromosomal alleles, but no segregation of *sd*/*ss*. All progeny of cross 1 were *sd*, and of cross 2 were *ss*.

In each of these crosses, thousands of zygotes were scored for their ability to germinate under the four conditions listed in Table 3. In cross 1, the results repeated those initially observed with the parental cross. Only zygotes matured and germinated on streptomycin agar gave viable progeny. In cross 2, however,

Table 3. *Percent germination of zygotes of F_1-backcrosses*

	Cross 1 *sd mt+* × *ss mt−*	Cross 2 *sd mt−* × *ss mt+*
Matured on S-agar, germinated on S-agar	98	0
Matured on S-agar, germinated on non-S-agar	77[1]	81[2]
Matured on non-S-agar, germinated on S-agar	0	34[1]
Matured on non-S-agar, germinated on non-S-agar . . .	0	100

[1] All progeny died after germination.

[2] All progeny survived after a delayed germination.

the results were quite different. Over 90% germination was achieved only with zygotes which had been matured and germinated in the *absence* of streptomycin; no germination was found with zygotes matured and germinated on streptomycin-agar. Some zygotes (34%) matured without streptomycin and did germinate on S-agar, but the progeny subsequently died. This result shows that *sd* property, which was dominant in the reciprocal cross, had already been partially lost in the zygotes of cross 2, not only in their progeny. However, the *sd* factor does have some influence in zygotes of cross 2 prior to germination, as shown by the recovery

of viable progeny from zygotes matured on streptomycin but germinated without it.

3. Crosses between dependent and resistant strains. It was of some interest to cross an *sd* strain with a strain carrying *sr*-500, since both factors show the same pattern of uniparental transmission. In the cross *sd mt* + × *sr*-500 *mt*—, as shown in Table 4 and Fig. 1, all progeny were *sd*, inheriting the determinant carried by the mating-type *plus* parent. In zygote germination studies, the same pattern of germination was observed as in crosses of the type *sd mt* + × *ss mt*—. The dominance of the *sd* factor was evident in the zygotes, from the death of all progeny germinated without streptomycin.

Table 4. *Classification of progeny from the cross: sdmt* + *act-r ms-s* × *sr*-500 *mt*— *act-s ms-r*

Progeny classes	Number and type of zygote segregation of chromosomal markers
(1) *sd act-r ms-s*; *sd act-s ms-r* .	2 Parental ditype
(2) *sd act-s ms-s*; *sd act-r ms-r* .	3 Nonparental ditype
(3) *sd act-s ms-r*; *sd act-s ms-s*; *sd act-r ms-r*; *sd act-r ms-s* .	17 Tetratype
	22

All tetrads showed 2:2 segregation of each pair of chromosomal alleles, but no segregation of *sd*/*sr*-500. All progeny were *sd*.

Recently, it has been possible to obtain some exceptional progeny which are double mutants, carrying both the *sd* and *sr*-500 factors. Studies of these strains will be reported subsequently.

II. Inheritance of the amplifier gene

A strain resistant to more than 2 mg of streptomycin per ml was obtained by plating a strain of mating-type *plus*, carrying the *sr*-500 factor, on agar

Table 5. *Inheritance of the amplifier gene*

	Number tetrads analyzed	Segregation ratios of streptomycin resistance level in each tetrad			
		Sens.	Resistant to		
			100 μg/ml	500 μg/ml	1500 μg/ml
(1) *sr*-500 *A mt*+ × *ss a mt*—	18	0	0	2	2
(2) *sr*-500 *A mt*+ × *sr*-500 *a mt*—	35	0	0	2	2
(3) *sr*-500 *A mt*— (F_1 of cross 2) × *sr*-100 *a mt*+ . .	67				
a)	9	2	0	0	2
b)	18	2	2	0	0
c)	40	2	1	0	1
(4) *ss A mt*+ (from cross 3b) × *ss a mt*—	32	all	0	0	0
(5) *ss A mt*+ (same strain as in 4) × *sr*-500 *a mt*—	30	all[1]			
(6) *ss A mt*— (from cross 3b) × *sr*-500 *a mt*+ . . .	32	0	0	2	2
(7) *ss A mt*— (same strain as in 6) × *sr*-100 *a mt*+	31				
a)	6	2	0	0	2
b)	7	2	2	0	0
c)	18	2	1	0	1
(8) *ss A mt*— (same strain as in 6) × *sd a mt*+ . .	3	0	0	2[2]	2[2]

In crosses 1—7 the progeny were also classified for four unlinked chromosomal gene pairs: *mt*+/*mt*—, *act-r*/*act-s*, *A*/*a*, *ms-r*/*ms-s*, which segregated regularly.

[1] Two exceptional tetrads were found in which *sr*-500 was transmitted to all progeny.

[2] All progeny, regardless of resistance level, are *sd*.

containing 2 mg of streptomycin per ml. Colonies appeared with a frequency of about 10^{-5} and one of these was chosen for genetic analysis.

The results of a series of crosses, listed in Table 5, show that the high resistance of this new strain is conferred by a chromosomal gene with unusual properties. This gene, called amplifier (*A*), does not increase the resistance level of *ss* cells, and therefore can only be detected in the presence of some *sr* or *sd* determinant. It interacts with *sr*-500 and with *sd* to heighten the resistance level, and similarly interacts with the chromosomal gene *sr*-100. The segregation of *A*/*a*, which is about 15 units from its centromere, has no effect upon the pattern of inheritance of either *sr*-500 or of *sd*.

III. Further studies of sr-500

In the course of an investigation of the process of mutation to *sr*-500, a large number of streptomycin-resistant strains were isolated and classified for resistance level. In all, about 10,000 clones were studied, most of them selected on agar containing 100 μg of streptomycin per ml. A few percent of these strains were found to be resistant to 500 μg/ml, the rest to only about 100 μg/ml. Very few strains of intermediate resistance level were encountered.

To date, 84 of these strains have been crossed, and complete correlation of resistance level and pattern of inheritance has been found. All of the 57 strains with low-level resistance, which were crossed with sensitive strains, have shown 2:2 segregation of resistance in tetrad analysis of progeny. Also, 27 strains resistant to 500 μg of streptomycin per ml have shown non-chromosomal inheritance, like that of the *sr*-500 factor initially described.

Most of the studies were carried out with strains of mating-type *plus*, in order not to lose any non-chromosomal factors obtained, in subsequent crosses. In addition, some mutants resistant to 500 μg/ml were isolated from a strain of mating-type *minus* to determine whether the mating type would influence the occurrence of this mutation. Mutants resistant to 500 μg/ml were isolated from the mating-type *minus* strain, with about the same frequency as from strains of mating-type *plus*, and the *sr*-500 factor was not transmitted in crosses of three of these strains. Thus, the same kind of mutation does occur in strains of both mating types, and the mutability of the factor is unrelated to its pattern of transmission.

To examine the effect of modifier genes from unrelated strains upon the uniparental pattern of inheritance, strains carrying *sr*-500 were outcrossed to other wild-type strains of *C. reinhardi*, obtained from G. M. Smith. A pair of strains of the two mating types from one zygote, and an unrelated strain of mating-type *plus* were tested. The results parallel those previously obtained: *sr*-500 was transmitted to all progeny when the *sr* parent was mating-type *plus*, and to none when it was mating-type *minus*. These results support the view that the uniparental pattern of transmission of these non-chromosomal factors is a general feature of the physiology of *C. reinhardi*, rather than a special property of a particular isolate.

IV. Studies of sr-1500

Clones resistant to 1500 μg of streptomycin per ml were isolated by plating cells of strain 4408 A (*sr*-500 *mt*—) on agar containing 1500 μg of streptomycin

per ml and incubating in the dark. This particular strain produces colonies from every cell under these conditions, although most strains carrying *sr*-500 do not do so.

In crosses of *sr*-1500 *mt*—$\times$ *ss mt*+, all progeny were uniformly sensitive. In crosses of *sr*-1500 *mt*—$\times$ *sr*-100 *mt*+, the progeny segregated 2:2 for resistance to 100 μg of streptomycin per ml. Thus, the *sr*-1500 factor shows the same uniparental pattern of transmission as do *sr*-500 and *sd*, so far as has been tested. As yet, we have not been able to obtain similar strains which are mating-type *plus*, for reasons that are probably not related to the mating type difference.

It is of some interest that *sr*-1500 is less stable than the other determinants, reverting back to the *sr*-500 level of resistance after many months in the absence of streptomycin. This process has not yet been studied in detail, but it has been observed that the *sr*-1500 property can be restored by replating such revertants under the original conditions.

C. Discussion

In this paper the genetic behavior of two chromosomal and three non-chromosomal determinants has been described. Each of these factors influences the cell's response to the drug streptomycin, and with the exception of *A* (amplifier), each acts independently of the others. The chromosomal factor *A* interacts with *sr*-100, *sr*-500 and with *sd* to amplify the resistance level of the cell. These phenotypic interactions have no effect upon the determinants themselves, as evidenced by the uniformity of segregation patterns in crosses with and without *A*.

The further studies of *sr*-500 presented here support and extend the initial report (SAGER 1954) that *sr*-500 (previously called *sr*-2) is a stable and permanent genetic constituent of the organism. Mutations to *sr*-500 have been obtained repeatedly in strains of both mating types. The uniparental pattern of transmission of these determinants has been found as well in crosses with two independent wild-type isolates of this species.

The *sd* determinant has provided material for a number of new findings and new lines of attack. In crosses of *sd mt*+ $\times$ *ss mt*— the dependence property was found to be dominant in the zygote, the presence of streptomycin being required for germination. In the reciprocal cross, there is no requirement for streptomycin. Indeed the drug is semilethal if present during the premeiotic maturation period, indicating that when the *sd* factor comes from the *mt*— parent, the *sd* property is lost before meiosis and germination occur. This finding shows that uniparental transmission is not the result of aberrant segregation, but rather that it involves the elimination or inactivation of the *sd* factor during zygote maturation.

These findings are of interest in relation to the physiology of streptomycin dependence. Zygotes receiving the *sd* factor from their *mt*+ parent have an absolute streptomycin requirement for germination; and the progeny from such a zygote cannot make even one division in the absence of the drug. After one sub-culture in the presence of streptomycin, however, the *sd* progeny strains can then be grown for many divisions in its absence before the requirement is expressed. Further studies of this phenomenon are in progress.

As yet, the non-chromosomal determinants have been identified only by their genetic behavior. In addition to their pattern of segregation in crosses,

extensive studies have been carried out on the origin of *sr*-500 (SAGER, unpublished). This factor has been found only in cells treated with streptomycin, and not as a clone of pre-existing mutants selected by the drug. These results suggest that the process of mutation from *ss* to *sr*-500 differs distinctly from that of spontaneous gene mutation, reflecting a significant difference in the determinants themselves.

At this stage in the investigation, one can only speculate about the nature of the non-chromosomal determinants. At the chemical level, it seems likely that these factors are or contain nucleic acid, whether they be extra-chromosomal DNA or RNA. This problem is the subject of current research efforts. At the cellular level, a number of hypotheses have appeared in the recent literature concerning the possible identity of non-chromosomal factors, and have already been briefly discussed in relation to the *sr* and *sd* determinants (SAGER 1960, 1961).

It has appeared of particular interest to try to distinguish critically between a determinant which replicates regularly regardless of its state of expression and has the structural stability of a permanent cell constituent, and a steady state system of the type postulated by DELBRUCK (1949) and POLLOCK (1953). The *sr*-500 factor has been maintained in cultures grown for several years under a wide variety of conditions in the absence of streptomycin, and in progeny cultures which are many sexual generations removed from the original parental strain which itself encountered streptomycin only once. Thus, the *sr*-500 factor is maintained in cells indefinitely in the absence of streptomycin. The fact that the uniparental pattern of transmission of *sr*-500 is not altered by the presence or absence of streptomycin, or of the modifying gene *A*, is further evidence of the separation between the genetic determinant and the phenotypic expression of *sr*-500. With the use of the double *sd sr*-500 recombinants recently obtained, attempts are in progress to test the independent segregation of *sd* and *sr*-500 and thereby in yet another manner to rule out the steady state hypothesis in this system.

Summary

Three non-chromosomal and two chromosomal genes which influence resistance to streptomycin are described. Each of the non-chromosomal factors, *sr*-500, *sr*-1500, and *sd*, exhibits uniparental inheritance, with all progeny receiving the factor when it is carried by the parent of mating-type *plus*, and none when it is carried by the mating-type *minus* parent. The streptomycin-dependence factor, *sd*, shows zygotic dominance when contributed by the mating-type *plus* parent, but not when coming from the mating-type *minus* parent, indicating that the uniparental transmission results from events occurring within the zygote early in maturation and well before meiosis. The chromosomal gene *A* interacts both with chromosomal and non-chromosomal genes at the biochemical level, but does not alter their patterns of inheritance.

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ANTIGENIC DIFFERENCES DUE TO THE INCOMPATIBILITY FACTORS IN *SCHIZOPHYLLUM COMMUNE**

By

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With 1 Figure in the Text

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I. Introduction

Tetrapolar incompatibility was first delineated by Kniep (1920, 1922) and has since been shown to be the characteristic pattern of sexuality in a majority of the higher Basidiomycetes (Whitehouse 1949, Quintanilha and Pinto-Lopez 1950, Raper 1953, Esser 1961). In *Schizophyllum commune*, as in all tetrapolar species, mating competence between mycelial strains is determined by two series of incompatibility factors, *A* and *B*.

Tetrapolarity is a prominent homogenic incompatibility system (Esser 1961). Mycelial compatibility follows only from heterozygosity of both *A* and *B* factors; incompatibility, with the formation of infertile heterokaryons, results from homozygosity of either *A* or *B* factors. Both *A* and *B* factors occur in the natural population in an extensive series of alternative and equivalent specificities (Raper, Krongelb and Baxter 1958). The factors are compound, each consisting of at least two subunits, α and β, that are separable by crossingover. Each subunit, in turn, has multiple alleles, and factor specificity is determined by an unique combination of specific subunits (Raper, Baxter and Middleton 1958, Raper, Baxter and Ellingboe 1960, Day 1959).

Although the major effects of the incompatibility factors have been known for decades and the genetics of the system has in recent years been worked out in considerable detail, the extensive literature on tetrapolar incompatibility provides no intimation of the mode of action of the incompatibility factors themselves. Further genetic studies can be expected to yield indicative evidence regarding the means by which the factors exert their precise and specific effects, but the problem is basically physiological rather than genetic, and its solution requires a physiological-biochemical approach.

The design of physiological or biochemical experimentation directed toward the elucidation of factor function presented difficulties because of the paucity of relevant information. The following reasoning, however, appeared applicable. The incompatibility factors must exert their control by means of specific chemical substances. Whatever the nature of the controlling substances, whether essential components or inhibitors, specific proteins, particularly enzymes, must be involved in the sequence of events leading to the fulfillment or inhibition of the function due to either *A* or *B* factors. A differentiation of specific proteins correlated either with specific incompatibility factors or with the four basic types of combinations between factors, i.e., heterozygous for *A* and *B*, heterozygous for *A* and homozygous for *B*, etc., thus seemed to provide the most reasonable hope for the clarification of the system. Since

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such proteins might be expected to be present in minute amount, the highly specific and extremely sensitive methods of immunology were clearly indicated as the experimental tool of first choice. The use of highly isogenic strains was considered necessary to minimize differences in constituent proteins except for those attributable to the incompatibility system. Serological methods were previously used by one of us (ESSER 1959) to demonstrate alteration of the specificities of proteins correlated with heterogenic incompatibility in the Ascomycete, *Podospora anserina*.

In the present paper are given the results of a preliminary serological comparison of extracts of total protein of two isogenic, compatible homokaryons and of the dikaryon established by their mating.

II. Materials and Methods

The two homokaryotic strains, $A^{41}_{1-1}\,B^{41}_{3-2}$ and $A^{43}_{4-6}\,B^{43}_{1-1}$, used in this study were provided by Dr. A. H. ELLINGBOE, who had backcrossed strains of the latter mating type with a strain of the former for ten successive generations. The choice of strains of these particular mating types was predicated on double heterozygosity of their *A* factors and of their *B* factors; from these two strains, other strains that would provide all known basic types of inter-factor (*A vs B*) and intra-factor (*A vs A*, *B vs B*) combinations could be generated via recombination.

Two nutrient media have been used: complete medium solidified with agar and the liquid, minimal medium of MILES, LUND and RAPER (1956), to which trace elements were added.

Mycelia to furnish extracts of proteins were grown in aerated, liquid cultures (8.5 l in 10 l bottles). Inoculum for each bottle was the macerated contents of a 3—5 day, 50 ml liquid culture. The homokaryotic mycelia were harvested when 4—5 days old; dikaryotic mycelia were harvested when only 2—3 days old, however, in order to avoid the large quantities of polysaccharide that later formed. The mycelia were harvested, twice resuspended in distilled water, pressed dry in pads of absorbent paper, and stored at —14° C.

To release the constituent proteins, the mycelium was hard-frozen in liquid nitrogen and disrupted in a HUGHES press block (HUGHES 1951), chilled on dry ice, with a pressure of 40—60 kg/cm².

The disrupted mycelium was suspended in three times its volume of 0.1 M phosphate buffer + 0.15 M NaCl and centrifuged for 20 min at 20.000 X G to remove all particulate matter. To the resulting supernatant was added 0.14 volumes of freshly prepared 1.5% solution of protamine sulfate to precipitate nucleic acids, and the precipitate was removed by centrifugation for 20 min at 20,000 X G. The protein was then precipitated in 90%-saturated $(NH_4)_2SO_4$, centrifuged for 10 min at 20,000 X G, taken up in a small volume of buffer-saline, and exhaustively dialyzed against buffer-saline. The protein-content of these antigenic preparations ranged from 15 to 30 mg/ml. All preparations were performed at or near 0° C, and storage was at —14° C; merthiolate was added to all samples of antigens and antisera at a concentration of 1:10,000.

The method of LOWRY et al (1951) was routinely employed for the determination of protein; protein-content was roughly determined upon occasion by differential absorption at 260 and 280 $m\mu$.

Rabbits were used for the production of antibodies. Protein precipitated by $(NH_4)_2SO_4$ served in most cases as antigen for injection; disrupted mycelium and untreated mycelial extracts were found to be less effective as antigens. Various techniques and schedules of injection were tested: simple solution of protein and alum-precipitated protein administered intervenously and protein emulsified in FREUND's complete adjuvant (cf. COHN 1952 for references) administered subcutaneously. The latter provided the most active antisera that were obtained. Relatively large doses of antigen were found to be necessary, and usable antisera have resulted only from the injection of total doses of 200—300 mg protein.

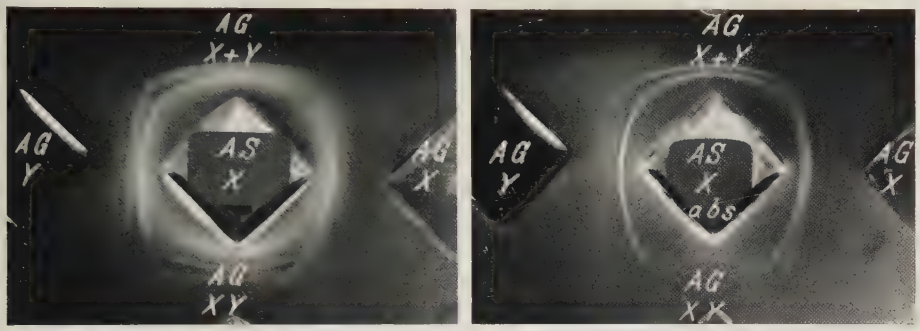
Antiseral activity was roughly determined by ring tests in small tubes with equal volumes (0.2 ml) of antigen and undiluted and diluted antiserum. Comparative tests of antigen-antibody reactions were made in agar plates according to the methods described by OUCHTERLONY and OUDIN (cf. OUDIN 1952 for references).

III. Results

Antisera were prepared against antigens of homokaryon $A^{41}B^{41}$ (four rabbits), homokaryon $A^{43}B^{43}$ (one rabbit) and the dikaryon ($A^{41}B^{41} + A^{43}B^{43}$) (one rabbit). The titers of antisera have not been greater than $^1/_8$ or $^1/_{16}$. Each antiserum has been tested against the separate antigens of the two homokaryons, the mixed antigens of the two homokaryons, and the antigen of the dikaryon; the arrangement of antiserum and antigens in these tests is shown in Fig. 1.

Antigens and antisera are subsequently identified in respect to origin as homo- and dikaryotic antigens and antisera, respectively.

The agar diffusion tests have repeatedly demonstrated two essential facts: (a) the close similarity—in most cases no differences could be seen—between the



a b

Fig. 1a and b. Agar diffusion tests of antigens derived from homo- and dikaryotic mycelia with unabsorbed and absorbed antiserum against antigen of homokaryon $A^{41}B^{41}$. a Unabsorbed antiserum. b Antiserum absorbed with antigen of dikaryon. *AS* antiserum; *AG* antigen; *X* homokaryon $A^{41}B^{41}$; *Y* homokaryon $A^{43}B^{43}$; *XY* dikaryon $A^{41}B^{41} + A^{43}B^{43}$

antigenic activities of the two homokaryotic mycelia and (b) several striking differences between the antigenic activities of the homokaryons and those of the dikaryon.

In all tests against either homo- or dikaryotic antisera, there was a heavy, poorly defined, and probably multiple, band of precipitate common to all four antigens (Fig. 1a). Since in no case, against any of the antisera, has any discontinuity or crossing of bands been observed in the regions between the mixture of homokaryotic antigens and the two separate homokaryotic antigens, there appears to be no constant difference in their basic activities. The homokaryons are accordingly considered together in comparison with the dikaryon. Aside from the reaction(s) common to all antigens, the reactions of the homo- and dikaryotic proteins against homo- and dikaryotic antisera may be approximately described as follows.

Homokaryotic antisera.—The four homokaryotic antisera, three against $A^{41}B^{41}$ and one against $A^{43}B^{43}$, have given remarkably constant patterns of reactions with the four standard antigens. Opposite the homokaryotic antigens and the mixture, there occur two bands that are usually quite distinct: the outer of these, sometimes modified in one or the other of the homokaryotic positions, is clearly peculiar to the homokaryons; the inner band, very constant elsewhere, appears to connect with a highly modified band in the dikaryotic position. Opposite the dikaryotic antigen, two strong bands are evident in addition to the

modified continuation of one homokaryotic band; one of these is clearly peculiar to the dikaryon, while the other turns towards the homokaryotic antigens and may connect with minor and undetected activities there.

Dikaryotic antiserum.—The pattern of reactions with the dikaryotic antiserum very strongly resembles that described above. There are, however, three significant points of difference: (a) opposite the dikaryotic antigen, there are three well separated and distinct bands, each clearly crossing all bands of the homokaryons, (b) the homokaryotic bands are weaker as compared to those of the dikaryon, and (c) the inner homokaryotic band is now continued through the dikaryotic position as a narrow, though faint, unmodified band.

These patterns of reaction are summarized in the Table.

An attempt was made with an antiserum against each of the three mycelia to absorb with heterologous antigen and thus to remove common antibodies. Within the time and with the materials available, this was successful in only one case: an antiserum against homokaryon $A^{41}B^{41}$ absorbed with antigen from the dikaryon (1 ml antiserum + 0.2 ml antigen containing 3.1 mg protein, for 48 hours). The antigen-antibody reactions that remained (Fig. 1a and b) clearly confirmed certain of the differences that had earlier been observed with unabsorbed antisera. The heavy, ill-defined component(s) common to all antigens and the distinct bands peculiar to the dikaryon were entirely absent. There remained only two bands: an outer band, peculiar to the homokaryons but strongly modified with one (light, diffuse band at left, Fig. 1b), and an inner band, distinct with the homokaryons but connecting with a diffuse, highly modified reaction with the antigen of the dikaryon (at bottom, Fig. 1b).

Table. *Comparative reactions of antigens derived from homo- and dikaryotic mycelia to their respective antisera*

	Antisera	
	Homokaryotic	Dikaryotic
Bands common to all	1*	1
Bands peculiar to both homokaryons	1**	1
Bands peculiar to homokaryons $A^{41}B^{41}$	—	
Bands peculiar to homokaryons $A^{43}B^{43}$	—	1
Bands peculiar to dikaryon	2—3	3

* Modified in dikaryon.
** Sometimes modified in one homokaryon.

A quantitative precipitation reaction between antiserum against $A^{43}B^{43}$ and antigens of $A^{41}B^{41}$, $A^{43}B^{43}$, and the dikaryon revealed no significant differences in the total reaction-products with the three different antigens. This result confirmed the impression that specific proteins correlated with the activity of the incompatibility factors represent only a very small portion of the total protein of the mycelia.

IV. Discussion and Conclusions

The results detailed above demonstrate unquestionable *differences in the proteins of two homokaryons and the dikaryon formed by their mating in a typical tetrapolar Basidiomycete, Schizophyllum commune.* Since the two homokaryons are highly isogenic for all other genetic characters, such specific protein-differences may be reliably considered as *reflecting biochemical activities of the incompatibility factors per se.* The present data, however, are far too meager to afford any

indication of the actual mode of action of the incompatibility factors, and no precise interpretation of the results is now possible. The significance of these preliminary observations lies rather in the demonstration that serological methods can be used for an eventual analysis of the physiological-biochemical basis of the activity of the incompatibility factors.

One observation regarding the patterns of interaction deserves brief consideration: the essential similarity of the reactions of all antigens to antisera of the two types, homo- and dikaryotic, i.e., the occurrence of activities in each mycelial type with heterologous antiserum. This effect might result from either of two causes: (a) Only minor alterations of the specificities of proteins result from the interaction of heterozygous *A* and *B* factors, and certain of the altered proteins remain cross-reactive with antibodies elicited by the originals. This was earlier found to be the case in *Podospora* (Esser 1959). (b) The two mycelial types may elaborate essentially the same species of proteins, and only their relative abundances are changed by the interaction of heterozygous factors.

To resolve such uncertainties and to permit a fruitful extension of the work, the methodologies employed must be considerably refined. When these necessary methodological prerequisites can be fulfilled, an extension of the comparative serological examination to include factor-mutations, factor-suppressors, and a wider array of factor combinations, e.g., common *A* and common *B* heterokaryons and the substitution of subunits to provide singly heterozygous *A* and *B* factors, should yield the information necessary for a critical choice between several possible modes of primary biochemical action of the incompatibility factors (Raper and Parag, to be published elsewhere).

Summary

A serological comparison of proteins extracted from a dikaryon and from its isogenic, homokaryotic component strains demonstrated differences that are attributable to tetrapolar incompatibility in the Basidiomycete, *Schizophyllum commune*.

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BASTARDIERUNGSVERSUCHE IN DER GATTUNG *STREPTOCARPUS* LINDL.

IX. NEUE UNTERSUCHUNGEN ÜBER DIE GESCHLECHTSBESTIMMUNG BEI *STREPTOCARPUS*

Von

Friedrich Oehlkers

Mit 3 Textabbildungen

(Eingegangen am 8. September 1961)

A. Einleitung

Auch über die Geschlechtsbestimmung wurden neue Untersuchungen angestellt. Die Notwendigkeit dazu ergab sich aus dem Befund der Arbeit IV, daß sich *Str. polyanthus* gegenüber *Str. wendlandii*[1] und *Str. comptonii* anders verhalten hat als *Str. rexii* bzw. *rexii* Gartenform. So erschien es wünschenswert, alle erreichbaren *Streptocarpus*-Formen mit *Str. wendlandii* oder *Str. comptonii* zu kreuzen. Inzwischen ist eine Arbeit von Lawrence (1958) erschienen, in der sich im Grundsätzlichen eine vollkommene Übereinstimmung mit meinen Resultaten findet. Allerdings ist es so, daß Lawrence gewöhnlich nur F_1-Bastarde heranzieht; infolgedessen stellt er (1958, S. 345, Tabelle 7) drei Klassen verschiedener Plasmone auf. Als erste faßt er *Str. wendlandii* und *comptonii* zusammen, durchaus in Übereinstimmung mit mir, als zweite *Str. polyanthus*, *gracilis*, *haygarthii* und *johannis*. Die dritte enthält *Str. rexii* mit einer ganzen Reihe anderer Formen dazu. Es sind das *Str. cyaneus*, *gardenii*, *parviflorus*, *pole-evansii*, *grandis*, *dunnii* und *michelmorei*. Er referiert meine an *Str. polyanthus* gewonnenen Resultate im einzelnen und teilt mit, ich hielte diese Art für eine intermediäre Form zwischen *Str. wendlandii* und *rexii*. Man kann diese Meinung durchaus vertreten, indessen ist es fraglich, ob man *Str. haygarthii* dann noch in diese Gruppe einordnen darf. Die Gründe, weswegen das mindestens zweifelhaft ist, sollen unter anderem in der folgenden Arbeit auseinandergesetzt werden.

B. Material und Methoden

Das Material ist dasselbe, wie das in der Serie meiner *Streptocarpus*-Arbeiten in Nr. VI beschriebene. Neu hinzu kommt noch *Str. haygarthii*, *gracilis* und *vandeleuri*, Formen, die ich ebenso wie alle anderen der Freundlichkeit von Dr. Lawrence verdanke. *Str. haygarthii* und *vandeleuri* sind unifoliate Typen, wobei bei *vandeleuri* das Anthocyan in der Blattunterseite nicht vorhanden ist. Infolgedessen zeigt *Str. vandeleuri* ein helles Grün, weil der

[1] Der Einheitlichkeit halber schreibe ich nunmehr so wie auch Lawrence alle Artbezeichnungen mit kleinen Anfangsbuchstaben, unabhängig davon, ob sie von Eigennamen abgeleitet sind oder nicht.

dunkle Untergrund der anthocyanhaltigen Epidermis fehlt. *Str. haygarthii* hat recht hohe Infloreszenzen, aber sehr kleine Blüten. Es ist nicht ganz einfach, mit dieser Form zu experimentieren (vgl. Abb. 1). *Str. randeleuri* besitzt große, aber relativ festgeschlossene, ein wenig röhrenförmige Blüten, die rein weiß sind, jedoch einen deutlichen gelben Fleck im Mittelfeld der Blüte haben (vgl. Abb. 2). *Str. gracilis* hat im vegetativen Bereich eine gewisse Ähnlichkeit mit *Str. comptonii*. Diese Form gehört zu denjenigen, die LAWRENCE 1958 als „subunifoliate" bezeichnet hat (vgl. Abb. 3). Das soll heißen: Wenn die Pflanzen älter werden, entwickeln sie von ihrer Basis aus noch zusätzlich 1—2 Blätter. Die Blüten von *Str. gracilis* sind hell und relativ groß; sie stehen in nicht allzu reichblütigen Infloreszenzen.

Methodisch halte ich es für notwendig, sich nicht allein auf die F_1 zu beschränken, sondern wenn irgend möglich Nachkommen aus Selbstbestäubungen sowie Rückkreuzungen der Bastarde mit dem väterlichen Elter aufzuziehen, in einzelnen Fällen verfährt LAWRENCE auch so. Es könnte sonst geschehen, daß rezessive Gene durch das plasmoneigene Genom unterdrückt, überhaupt nicht zur Entfaltung im fremden Plasmon gelangen. Auf diese Weise läßt sich tatsächlich ein sehr tiefgreifender Unterschied zwischen *Str. polyanthus* und *haygarthii* konstatieren.

Abb. 1. *Streptocarpus haygarthii*

Bevor wir zur Betrachtung unserer experimentellen Resultate übergehen, sei noch kurz darauf verwiesen, daß es sich bei der plasmonisch bedingten Geschlechtsbestimmung nicht allein um eine Verweiblichung der Zwitterblüte handelt, also um „male sterility" nach der Bezeichnung von LAWRENCE. Es findet sich vielmehr in den reziproken Bastarden mit *Str. wendlandii* und *comptonii* als Vater bei völlig normaler Ausbildung des Staubblattkreises eine Einschränkung in der Fertilität des Gynaeceums (vgl. OEHLKERS 1938, S. 375—377). Trotzdem sind in der Tabelle 2 allein die verschiedenen Grade der Verweiblichung eingetragen, weil sie sich — am einfachsten ablesbar — durch den Antherenzustand der Blüten unterscheiden. Als Gedächtnisstütze geben wir hier noch einmal diese Zustände an, wie wir sie früher (OEHLKERS 1938) aufgestellt haben. Gruppe Nr. 1 bedeutet vollfertile Antheren, wobei im Laufe der Staubblattentwicklung die Antherenspitzen durch verschränkte Haare miteinander in Zusammenhang stehen. Nr. 2 bedeutet getrennte Antheren. Falls die ganzen Staubblätter eine nicht genügende Längenentwicklung haben, so daß sie sich in der Antherenspitze nicht mehr berühren, kommen auch die Haarverschränkungen nicht mehr zustande. Nr. 3 bedeutet getrennte und zu kleine Antheren. Dabei hat sich also die Minderentwicklung des Staubblattkreises schon verstärkt. Nr. 4 bedeutet Staminodien mit leichten Erweiterungen, Nr. 5 Staminodien ohne besonderen Charakter, Nr. 6 Staminodien fehlen, Nr. 7 Staminodien mit Narbenpapillen, Nr. 8 Staminodien mit Narbenpapillen und Samenanlagen an der Basis, Nr. 9 real ausgebildeter Nebenfruchtknoten, die freilich deshalb, weil der Fruchtknoten zwei Blätter umfaßt, die Staubblätter dagegen je aus einem Blatt hervorgegangen sind, offen sind[1]. Nur auf der einen der Außenflächen befindet sich eine Fruchtknotenwand mit einer normalen Epidermis, auf der anderen Seite hingegen ist eine offene Placenta-Anlage mit zahlreichen Samenanlagen.

[1] Dabei ist folgendes von Interesse. GOTTSCHALK (1960, 1961) hat unter den zahlreichen Mutanten von *Pisum sativum*, die er durch Röntgenbestrahlung erhielt, auch zwei gefunden, die sich im Geschlechtsverhalten von der Normalform unterscheiden. Bei einer davon, der

Die Bonitur bezieht sich hier auf einzelne Pflanzen. Selbstverständlich sind eine größere Anzahl von Blüten beurteilt worden, und danach stellt die einzelne Pflanze einen Durchschnittswert dar; denn daß alle Blüten einer Pflanze völlig gleich ausgebildet würden, ist selten. Bei den zwittrigen Ausgangsformen ist es freilich stets der Fall. Im übrigen ist die Anordnung der Kreuzungen in Tabelle 2 so gewählt, daß sie gleichzeitig in ihrer Aufeinanderfolge angeben, wie intensiv die einzelnen Formen auf das *wendlandii*-Plasmon ansprechen.

Mutante 192, findet er eine vollkommene Verweiblichung des Androeceums, ähnlich wie das hier durch Plasmoneinwirkung zustande gekommen ist. Von besonderem Interesse ist nun, daß die Nebenfruchtknoten, die aus den je einblättrigen Staubblättern entstehen, normale geschlossene *Pisum*-Fruchtknoten darstellen, weil ja *Pisum* auch normalerweise die Fruchtknoten aus einem Blatt hervorbringt. Wir sehen also im Vergleich mit *Streptocarpus*, daß die Herkunft des normalen Gebildes aus ein oder zwei Blattanlagen entscheidend für seine Ausgestaltung und offenbar vollkommen unabhängig von der eigentlichen Sexualreaktion ist. Im übrigen fanden wir auch unter unseren Röntgenmutanten von *Streptocarpus* eine solche mit total verweiblichten Blüten, deren dominantes Korrelat anscheinend völlig unabhängig ist von der durch das Plasmon beeinflußbaren sexuellen Ausgestaltung. Wir werden später in einer anderen Arbeit auf diesen Typ genauer eingehen.

Abb. 2. *Streptocarpus gracilis*

Abb. 3. *Streptocarpus vandeleuri*

C. Experimentelle Ergebnisse

1. Untersuchungen über Str. haygarthii

Wie aus unserer Tabelle 1 hervorgeht, haben wir nicht nur bei den Kreuzungen von *Str. haygarthii* mit *wendlandii* und reziprok, sondern auch mit *Str. comptonii* und reziprok und sehr vielen anderen Formen immer nur normale, im männlichen

Tabelle 1

Nr.	Kreuzung	Antherenzustand
181 1953/31	*Str. (wendlandii × haygarthii)*	1
182 1953/32	*Str. (haygarthii × wendlandii)*	1
183 1950/52	*Str. (comptonii × haygarthii)*	1
184 1950/51	*Str. (haygarthii × comptonii)*	1
185 1953/69	*Str. (rexii albus × haygarthii)*	1
186 1953/68	*Str. (haygarthii × rexii albus)*	1
187 1950/59	*Str. (haygarthii × polyanthus)*	1
188 1950/60	*Str. (polyanthus × haygarthii)*	1
189 1950/64	*Str. (michelmorei × haygarthii)*	1
190 1953/84	*Str. (haygarthii × solenanthus)*	1
191 1953/85	*Str. (solenanthus × haygarthii)*	1
192 1954/196	*Str. (wendlandii × haygarthii) × haygarthii*	1
193 1954/195	*Str. (haygarthii × wendlandii) × wendlandii*	1
194 1954/108	*Str. (haygarthii × rexii albus) × rexii albus*	1
195 1954/109	*Str. (haygarthii × rexii Basel) × rexii Basel*	1
196 1951/120	*Str. (comptonii × haygarthii)* × selbst	1
197 1951/119	*Str. (haygarthii × comptonii)* × selbst	1
198 1951/137 1952/158	*Str. (comptonii × haygarthii) × haygarthii*	1
199 1951/133 1952/156	*Str. (haygarthii × comptonii) × comptonii*	1
200 1951/117 1952/161	*Str. (polyanthus × haygarthii) × haygarthii*	1
201 1951/116	*Str. (haygarthii × polyanthus) × polyanthus*	1

Es wurden je Nachkommenschaft 50—100 Pflanzen aufgezogen.

wie weiblichen Geschlecht vollfertile Pflanzen gefunden. Weiterhin schien es uns zweckmäßig zu sein, Rückkreuzungen mit dem väterlichen Elter sowie Selbstbestäubungen aufzuziehen. Da es sich um den bisher ersten und einzigen Fall eines solchen Verhaltens handelt, wurde mit besonderer Sorgfalt vorgegangen und zahlreiche F_1-Bastarde sowie Nachkommen von Selbstbestäubungen aufgezogen. Und in allen Fällen, wie die Tabelle 1 zeigt, findet man einen Antherenzustand 1, also vollkommene Normalität, auch im mehrfach zu Kreuzungen verwendeten weiblichen Geschlecht in den *haygarthii*-Kreuzungen. Obwohl wir gewohnt waren, daß die im Genom liegenden Erbfaktoren, die sich auf die Geschlechtsbestimmung beziehen, bei den Rückkreuzungen im Verhältnis von 1:1 spalten, sich also gut bei einer Aufzucht von 30—40 Pflanzen feststellen lassen, haben wir dennoch im Fall der Kreuzungen mit *Str. haygarthii* stets eine reichliche Anzahl von Pflanzen aufgezogen; in den meisten Fällen waren es 100. Niemals ist uns eine Abweichung vom normalen Zustand einer Zwitterblüte aufgefallen. Auch kompliziertere Mehrfachkreuzungen sind gemacht, aber hier nicht mit aufgeführt worden. Sie zeigen einerseits an, daß sich das Plasmon von *Str. comptonii*, obwohl das Genom von *Str. haygarthii* hineingelangt ist, dennoch

genauso wie sonst gegenüber *rexii* verhält. Abgesehen davon scheint es uns, als ob die Kreuzung zwischen *haygarthii* einerseits und *comptonii* und *wendlandii* andererseits ein gutes Material für die endgültige Auflösung der Frage nach dem Übertritt des Plasmons durch den Pollenschlauch darstellt. Freilich bedarf das noch einiger Kreuzungen und Aufzuchten, so daß wir erst in einer späteren Arbeit darauf zurückkommen können.

2. *Kreuzungen mit sonstigen Streptocarpus-Arten*

Wir haben alle diese Kreuzungen in Tabelle 2 zusammengestellt, und zwar so, daß sie in ihrer Aufeinanderfolge die Intensität angeben, in der sie sich von *Str. wendlandii* unterscheiden. Die ersten drei dieser Formen, *Str. gracilis*, *Str. baudertii* und *Str. meyeri* gehören mit denen zusammen, für die Lawrence (1958, S. 345, Tabelle 7) die Klasse 2 zusammengestellt hat. Alle drei nämlich zeigen in der F_1 Antherenzustand 1, d. h. vollfertile und normale Antheren. Und weiterhin besitzen sie auch ein normales Gynaeceum, wie die Rückkreuzungen bzw. Selbstbestäubungen zeigen. Freilich unterscheiden sie sich auch alle drei von *Str. haygarthii:* bei den Rückkreuzungen und Selbstbestäubungen zeigen sie eine Aufspaltung, so daß sie sich ähnlich verhalten wie *Str. polyanthus* (vgl. Oehlkers 1941). *Str. gracilis* zeigt das Phänomen am geringsten, unter 44 Pflanzen der Rückkreuzung mit *gracilis*, also mit dem väterlichen Elter, zeigen immerhin 27 Pflanzen von 44 den Antherenzustand 1, 9 den Antherenzustand 2. Auch bei den letzteren dürfte noch relativ normaler Pollen ausgebildet werden. Ferner findet sich bei 8 Pflanzen ein Antherenzustand 3—5. Bei diesen wenigen also haben wir es direkt mit einer Sterilität im männlichen Geschlecht zu tun, jedoch treten diese sterilen Pflanzen keineswegs in Zahlenverhältnissen auf, die — wie bei *polyanthus* — eine statistische Sicherung als Mendelzahlen zuließen. *Str. gracilis* steht also auf der Grenze zwischen dem Verhalten von *Str. polyanthus* und dem völlig abweichenden von *Str. haygarthii*. Im übrigen sei noch hinzugefügt, daß mit *Str. gracilis* schwer zu arbeiten ist, die Pflanzen sind nicht gut wüchsig, und außerdem setzen die Kreuzungen schlecht an.

Die nächste Kreuzungsserie in Tabelle 2 bezieht sich auf das interessante Verhalten von *Str. baudertii*. Zunächst ist ohne weiteres deutlich, daß *baudertii* ebenfalls in die von Lawrence aufgestellte Klasse 2 hineingehört, weil die F_1 in reichlicher Anzahl aufgezogen mit *Str. wendlandii* als Mutter allein den Antherenzustand 1 aufweist, sich also völlig normal verhält. Anders steht es mit den Rückkreuzungen. Hier ergibt sich sofort in der ersten Rückkreuzung mit *baudertii* als väterlichem Elter eine Streuung über die gesamte Skala von 1—9.

Bei allen Vorkommnissen, die wir als plasmatische Vererbung bei *Streptocarpus* bezeichnen können, hat es sich stets um eine bestimmte Reaktion eines karyotischen Gens in den verschiedenen Plasmonen gehandelt. Daß dabei wirklich karyotisch lokalisierte, also mendelnde Gene vorliegen, ist nicht immer leicht zu erweisen. Ich erinnere nur an die Schwierigkeiten, die ich mit dem anscheinend so einfachen Phänomen der Blütenschlitzung hatte. Leider ist es im Fall des *Str. baudertii* nicht anders. Wenn wir die erste Rückkreuzung des *wendlandii-baudertii* Bastardes (Nr. 81) mit dem Verhalten von *Str. polyanthus* vergleichen, dann kann man auch hier eine Aufspaltung im Verhältnis 1:1 konstruieren. Rechnet man alles was links von der Antherenzustandsgruppe 4, in der sich keine

Tabelle 2

Nr.	Kreuzung	Antherenzustand des weiblichen Elters der letzten Rückkreuzung	Antherenzustand								Anzahl der Pflanzen
			1	2	3	4	5	6	7	8,9	
202 1954, 14	*Str. (wendlandii × gracilis)*		22								22
203 1954, 13	*Str. (gracilis × wendlandii)*		21								21
204 1954, 20	*Str. (wendlandii × gracilis) × gracilis*		27	9	4	3	1				44
205 1956, 25, ♀ 3	*Str. (wendlandii × gracilis)* × selbst		8	3	1			1			13
206 1954, 19	*Str. (gracilis × wendlandii) × wendlandii*		34								34
80[1]	*Str. (wendlandii × baudertii)*		45								45
207 1955, 143	*Str. (baudertii × wendlandii)*		44								44
81	*Str. (wendlandii × baudertii) × baudertii*		17	7	1		1	16	4	1	47
208 1955, 129, ♀ 19	*Str. (wendlandii × baudertii) × baud. × baud.*	1	7	3	4	1	1	20	1	2	39
209 1955, 129, ♀ 20	*Str. (wendlandii × baudertii) × baud. × baud.*	5						22	1	1	24
210 1955, 129, ♀ 18	*Str. (wendlandii × baudertii) × baud. × baud.*	6	12	2	3	4	5	15	1		42
211 1955, 129, ♀ 25	*Str. (wendlandii × baudertii) × baud. × baud.*	6	11	5	6	5	4	8			39
212 1955, 129, ♀ 1	*Str. (wendlandii × baudertii) × baud. × baud.*	6	8	5		5	6	13			37
213 1955, 129, ♀ 5	*Str. (wendlandii × baudertii) × baud. × baud.*	6						41	2	1	44
214 1955, 129, ♀ 35	*Str. (wendlandii × baudertii) × baud. × baud.*	8						36	5	2	43
215 1955, 129, ♀ 33	*Str. (wendlandii × baudertii) × baud. × baud.*	9	1				5	21	9	4	40
84	*Str. (wendlandii × meyeri)*		38								38
216 1954, 46	*Str. (meyeri × wendlandii)*		7								7
85	*Str. (wendlandii × meyeri) × meyeri*							26	2	8	36
217 1956, 305	*Str. (wendlandii × meyeri) × meyeri × meyeri*							3			3
88	*Str. (wendlandii × michelmorei)*				30						30
218 1955, 22	*Str. (michelmorei × wendlandii)*		25								25
89	*Str. (wendlandii × michelmorei) × michelmorei*				5	1	6	31	1	1	45
219 1954, 201, ♀ 3	*Str. (wendlandii × michelm.) × michelm. × michelm.*	4					9	30			39
220 1954, 201, ♀ 4	*Str. (wendlandii × michelm.) × michelm. × michelm.*	6					9	26	1		36
221 1954, 201, ♀ 6	*Str. (wendlandii × michelm.) × michelm. × michelm.*	6			2	4	29	35	1		71
222 1954, 201, ♀ 7	*Str. (wendlandii × michelm.) × michelm. × michelm.*	8					9	18			27
223 1955, 24	*Str. (wendlandii × rexii Basel)*						28				28
224 1955, 27	*Str. (wendlandii × rexii type)*						20				20
225 1955, 26	*Str. (rexii type × wendlandii)*		20								20

226 1955, 204	*Str. (rexii K 1275 × wendlandii)*				30									30
227 1956, 15	*Str. (wendlandii × rexii K 1275)*									36				36
228 1955, 134	*Str. (rexii K 1275 × wendlandii) × wendlandii* . .				18	2								20
229 1955, 152	*Str. (wendlandii × rexii K 1275) × rex. K 1275 × rexii K 1275*									35				35
230 1956, 324	*Str. (wendlandii × rexii K 1275) × rex. K 1275 × rex. K 1275 × rex. K 1275*									15	14	4		33
231 1954, 11	*Str. (wendlandii × vandeleuri)*								20					20
232 1954, 12	*Str. (vandeleuri × wendlandii)*				17									17
233 1957, 56	*Str. (vendeleuri × wendlandii) × wendlandii* . . .				11									11
234 1957, 149	*Str. (wendlandii × parviflorus)*							11						11
82	*Str. (comptonii × gardenii)*							1	17	5				23
83	*Str. (comptonii × gardenii) × gardenii*									12	13	23		48
235 1956, 209, b ♀	*Str. (comptonii × gardenii) × gardenii*									44				44
92	*Str. (wendlandii × montigena)*							12	3	3				18
236 1956, 87	*Str. (montigena × wendlandii)*				9									9
93	*Str. (wendlandii × montigena) × montigena* . . .									11	9	23		43
237 1956, 281, a ♀10	*Str. (wendlandii × montigena) × mont. × mont.* . .											26		26
238 1956, 281, b ♀9	*Str. (wendlandii × montigena) × mont. × mont.* . .											25		25
					432	36	56	47	173	523	64	121		1452

[1] Die allein mit einer laufenden Nummer bezeichneten Kreuzungen finden sich alle in Oehlkers (1957), Tabelle 3, unter anderen Gesichtspunkten aufgeführt.

Pflanzen vorfinden, zum *wendlandii*-Genom und das was rechts davon steht zu dem von *Str. baudertii*, so haben wir ein Zahlenverhältnis von 25:22. Das ergibt eine gute Übereinstimmung mit einem 1:1-Verhältnis: unter 47 Pflanzen wäre das theoretische Verhältnis 23,5:23,5, statistisch betrachtet sind die theoretischen und experimentellen Werte homogen.

Aus dieser Rückkreuzung Nr. 81 haben wir nun 8 Pflanzen ausgesucht, den Antherenzustand der betreffenden Blüte notiert und wiederum mit *Str. baudertii* rückgekreuzt. Das Resultat findet sich unter Nr. 208—215. Bei der Kreuzung unter Nr. 208, bei der die Ausgangspflanze, insbesondere die Ausgangsblüte, den Antherenzustand 1 besitzt, sollten wir eine Häufung in den Klassen 1, 2 und 3 erwarten. Das trifft aber nicht zu, vielmehr sehen wir das Maximum eindeutig bei 6 liegen. Beim Antherenzustand 5, Kreuzungsnummer 209, finden wir in der Tat eine einseitige Verschiebung nach der weiblichen Richtung. Freilich nur durch 24 Pflanzen. Sodann haben wir uns etwas genauer mit dem Antherenzustand 6 befaßt. Daß dieser eine unsichere Größe darstellt, ist wahrscheinlich. Wir finden nun dreimal hintereinander,

Nr. 210, 211 und 212, daß die Nachkommen der Pflanzen aus Antherenzustand 6 über die Skala bis 6 mindestens, eine sogar bis 7 streut. Erstaunlich und nicht ohne weiteres zu verstehen ist die Tatsache, daß sehr viele Pflanzen aus diesen Kreuzungen 210–212 sich im Antherenzustand 1 befinden, also normal fertil sind. Allein die Kreuzung Nr. 213 bringt ein einseitiges Verschieben der Pflanzen nach Gruppe 6–9, und die Ausgangspflanzen von den Nr. 214 und 215 mit Antherenzustand 8 und 9 geben in ihren Nachkommen eine weitgehend einseitige Verschiebung nach der weiblichen Richtung. Aber auch in diesen Fällen haben wir es noch mit einer starken Variabilität zu tun.

In diese Reihe, unmittelbar nach *Str. baudertii* und den darin angegebenen Zahlen, gehören nun die Kreuzungen mit *Str. polyanthus*, wie ich sie in meiner Arbeit „IV" (vgl. OEHLKERS 1941) dargestellt habe. *Str. polyanthus* gehört in die gleiche von LAWRENCE (1958, S. 345) angegebene Plasmon-Klasse 2, d. h. in unserer Sicht: Es sind das diejenigen Formen, deren F_1 mit *Str. wendlandii* und *comptonii* im männlichen Geschlecht völlig normal sind (Antherenzustand Nr. 2). In der Tat ließen sich sowohl von *Str. polyanthus* ♂ *wendlandii* wie auch von *Str. wendlandii* × *polyanthus* Nachkommenschaften aufziehen (Nr. 58, 1941, S. 170). *Str. wendlandii* ♂ *polyanthus* selbstbestäubt ergab zu wenig Pflanzen, um damit ein Zahlenverhältnis auszudrücken. Von den vorhandenen 22 Pflanzen haben nur 11 geblüht. Wichtiger war die Rückkreuzung mit dem väterlichen Elter (Nr. 60, 1941, S. 172) *Str. (wendlandii* ♂ *polyanthus)* × *polyanthus*. Dabei kamen 45 Pflanzen zur Blüte. Wir hatten eine vollkommen klare Spaltung: 22 Pflanzen hatten ein normal-fertiles Androeceum, 23 Pflanzen waren steril. Der entscheidende Unterschied zwischen *polyanthus* und *baudertii* besteht nun darin, daß hier so gut wie überhaupt keine Variabilität in der Ausprägung der Veränderung des Androeceums vorgekommen ist. Die allermeisten der dahin gehörigen Pflanzen besaßen Staminodien ohne besonderen Charakter.

Von der nächsten Gruppe, den Kreuzungen mit *Str. meyeri*, die ebenfalls in die Klasse 2 von LAWRENCE gehört, weil die F_1 normal ist, kann nicht allzuviel ausgesagt werden, weil nur relativ wenig Pflanzen zur Beobachtung kamen. Immerhin ist es erstaunlich, daß obwohl die F_1 eindeutig in die Gruppe 1 gehört, dennoch die Rückkreuzung mit der *meyeri* durchaus nach der weiblichen Seite hin tendiert. Im übrigen scheinen hier Fertilitätseinschränkungen mitzuspielen: Die zweite Rückkreuzung hat nur drei Pflanzen hervorgebracht. Ähnliche Verhältnisse finden wir bei der nächsten Kreuzungsgruppe, mit *Str. michelmorei*. In Übereinstimmung mit LAWRENCE gehört *Str. michelmorei* der Klasse 3 an. Bereits in der F_1 findet sich der Antherenzustand 3, d. h. getrennte und zu kleine, jedenfalls schon halbwegs sterile Antheren. Ähnlich wie bei *Str. baudertii* gibt es nun in der Rückkreuzung eine ziemliche Streuung. Bereits die erste Rückkreuzung variiert von Antherenzustand 3–9, die weiteren Rückkreuzungen sind nicht besonders aufschlußreich, sie zeigen ebenfalls eine recht erhebliche Variation an.

Die nächste größere Gruppe von Kreuzungen hat mehr oder weniger nur den Sinn nachzuweisen, daß meine nunmehr verwendeten *rexii*-Formen sich durchaus den bisher verwendeten anschließen. Interessant ist dabei das Verhalten von *Str. rexii K 1275*, einer rein weißen Sippe von LAWRENCE, die schon in der F_1, besonders aber nach der Rückkreuzung, durchaus stärker nach der weiblichen Seite variiert als die übrigen.

Von *Str. parviflorus* wurde nur eine Kreuzung mit *Str. wendlandii* aufgezogen, sie gehört also durchaus den Formen der Klasse 3 an, genau wie Lawrence angegeben hat. *Str. gardenii*, ebenfalls der Klasse 3 angehörig, ließ sich schlecht oder gar nicht mit meinen *wendlandii*-Typen kreuzen, wohl aber gut mit *comptonii*. Wir sehen hier einen immer stärkeren Ausschlag nach der weiblichen Seite; bei den Rückkreuzungen finden sich allein Angehörige der Gruppe 6—9. Endlich *Str. montigena* mit seinen Kreuzungen variiert schon ebenso stark wie *gardenii* in der F_1, um dann in den Rückkreuzungen schließlich ganz und gar in die Gruppe 9 überzugehen. Damit ist die Übersicht über unsere neueren Resultate beendigt.

D. Diskussion der Ergebnisse

1. Das Verhältnis von Genom und Plasmon bei der Geschlechtsbestimmung

Überblickt man nun die sämtlichen vorliegenden Resultate, sowohl aus meinen früheren Arbeiten als auch derjenigen von Lawrence (1958) mit den hier neu publizierten, so wird vollkommen klar ersichtlich, erstens, daß es sehr verschiedene Möglichkeiten gibt, in der ein fremdes Genom mit dem Plasmon von *Str. wendlandii* bzw. *Str. comptonii* fertig werden kann, und zum anderen, daß es stets das fremde Genom ist, das zu bestimmen hat, welch einen Anteil das *wendlandii*-Plasmon an der entscheidenden Geschlechtsbestimmung einnimmt. In Tabelle 1 sind die Kreuzungen mit *Str. haygarthii* aufgeführt. Betrachtet man zunächst diejenigen mit *Str. wendlandii* und *comptonii*, dann hat es den Anschein, als gehöre *Str. haygarthii* in die gleiche Plasmongruppe, in der sich *Str. wendlandii* und *comptonii* befinden. Freilich müßten dann die Kreuzungen mit allen den *Streptocarpus*-Arten, die Lawrence der Klasse 3 zugeordnet hat, ebensolche Sterilitätsverhältnisse im männlichen Geschlecht ergeben, wie das bei den Kreuzungen mit *Str. wendlandii* und *comptonii* als Mutter mit *Str. rexii* der Fall ist. Das trifft nun aber nicht zu, sondern alle Kreuzungen mit Pflanzen, die der Klasse 2 oder 3 angehören: *Str. polyanthus*, *rexii* und *solenanthus*, sowie deren F_2 bzw. Rückkreuzungen mit dem väterlichen Elter, ergeben ausschließlich und eindeutig im Geschlechtsverhalten völlig normale Pflanzen. Gerade diesen Befund haben wir mit besonderer Sorgfalt experimentell ausgearbeitet, so daß kein Zweifel daran bestehen kann, daß das Genom von *Str. haygarthii* weder irgendwie anders als normal mit dem Plasmon von *Str. wendlandii* und *comptonii* reagiert, noch daß es selbst ein Plasmon besitzt, das bei Einkreuzen von *Str. polyanthus* bzw. *rexii* oder *solenanthus* irgendwelche Sterilitätserscheinungen zeigte. Wir haben es also bei *Str. haygarthii* ohne Zweifel mit einem Grenzfall zu tun. In der Tat erbringt die nächste Tabelle 2 mit den an den Anfang gestellten Kreuzungen zwischen *Str. wendlandii* und *gracilis* durchaus den Nachweis dafür. *Str. gracilis* zeigt eine nahe Verwandtschaft mit *Str. haygarthii* und dessen Plasmon.

Nehmen wir zunächst einmal an, daß von dem männlichen Elter allein eine generative Zelle mit einem Genom in die weibliche Eizelle übergeht, die ebenfalls mit einem Genom und dazu noch mit reichlich Plasmon ausgestattet ist, dann sehen wir mit Deutlichkeit aus der Zusammenstellung dieser Kreuzungsabfolgen, daß es jeweils der männliche Elter, d. h. also das plamsmonfremde Genom (vgl. Michaelis 1957 und 1958) ist, welches den Charakter der Kreuzung bestimmt, etwas anders ausgedrückt: dafür sorgt, wieviel und was von dem *wendlandii*-Plasmon in der betreffenden Kreuzung zur Reaktion gelangt. Die Kreuzungen

mit *Str. haygarthii* (vgl. Tabelle 1) haben gezeigt, daß sogar die Möglichkeit einer vollkommen gleichmäßigen Reaktion besteht, so daß sich sämtliche fremde Genome, die sich überhaupt mit dem von *Str. haygarthii* vereinigen lassen, sich so benehmen, als säßen sie in ihrem eigenen Plasmon und vice versa. So geht es nun fort (Tabelle 2), bis zu der Kreuzung mit *Str. montigena*, bei der eine maximal abweichende Reaktion erfolgt, und zwar so, daß einerseits männlich sterile, andererseits in den reziproken Kreuzungen weiblich sterile Formen entstehen. Michaelis (1958) bezeichnet solche Erscheinungen als Folge verschiedener Plasmonempfindlichkeit der betreffenden Genome, und Caspari macht sogar davon eine „theory of plasmon sensitive genes". Warum ich diesen Ausdruck nicht verwende, habe ich eingehend 1952, S. 227—231 auseinandergesetzt. Ich möchte nur wiederholen, daß man prinzipiell ja jedem Gen eine „Plasmonempfindlichkeit" zuordnen muß, damit es überhaupt zu seiner Wirkung kommen kann. Damit ist das theoretisch Bedeutsame der Tabellen 1 und 2 hinsichtlich des Geschlechtszustandes zusammengefaßt.

2. Geschlechtsbestimmung und Blütenschlitzung

In meiner Arbeit von 1957 sind in Tabelle 3 eine Reihe von Kreuzungen aufgeführt, die sich auch in der hier vorliegenden Tabelle 2 wiederfinden; es sind das alle diejenigen, die dort lediglich mit einer laufenden Nummer bezeichnet sind. Da wir die laufenden Nummern aus der Arbeit VI bezogen haben, wird zunächst einmal deutlich, daß dort *Str. haygarthii* und *gracilis* fehlen. In der Tat zeigen Bastardnachkommen dieser Arten keine Spur von Blütenschlitzung. Sodann wird deutlich, daß — sieht man von *Str. gracilis* ab — Anfang und Ende der Tabellen übereinstimmen. Es beginnt die Tabelle 3 (1957) mit den Kreuzungen von *Str. wendlandii* und *baudertii* und endigt mit denen mit *montigena*. Zwischen diesen beiden Extremen findet sich jedoch eine völlige Nichtübereinstimmung hinsichtlich der Intensität von Blütenschlitzung und Geschlechtsbestimmung, was sich in der Reihenfolge der Anordnung zwischen beiden Extremen deutlich abzeichnet. So folgt auf *Str. baudertii* in der Blütenschlitzungstabelle (Tabelle 3, 1917) *Str. gardenii*, während in der Tabelle der Geschlechtsbestimmung auf *baudertii meyeri* folgt, sodann *michelmorei*. Schon in einer früheren Arbeit (1956) hatten wir dargelegt, daß zwar die Blütenschlitzung dann auftritt, wenn gleichzeitig auch eine plasmonisch bedingte Verschiebung des Geschlechts erfolgt, daß aber dennoch beide Phänomene nichts miteinander zu tun haben. Die Tatsache, daß sich eine phänotypische und genotypische Beeinflussung bei Geschlechtsbestimmung durch die Temperatur findet (1956), jedoch keine hinsichtlich der Blütenschlitzung, und außerdem hier die Intensität der Erscheinung in beiden Fällen eine andere ist, erhärtet die schon 1956 vorgetragene Meinung.

3. Die Variabilität der Geschlechtsausprägung

Zunächst sei darauf aufmerksam gemacht, daß ich bereits einmal über eine so weitgehende Variabilität berichtet habe, wie sie in etwa in den Kreuzungen von *Str. wendlandii* mit *baudertii* zu finden ist. Die Variabilität ist bei Oehlkers (1938, S. 365) eingetragen, es handelt sich dabei um eine etwas komplizierte

Kreuzung: Nr. 30, *Str.* (*wendlandii* × *rexii* Gartenform weiß) × (*rexii* Gartenform weiß × *wendlandii*). Diese Kreuzung entspricht der Selbstbestäubung von *Str. wendlandii* × *rexii* Gartenform, die darum direkt nicht möglich ist, weil hier in der F_1 Antheren nicht ausgebildet werden. Damals habe ich keinen besonderen Wert auf die Variabilität gelegt, schon deshalb, weil mir bekannt war, worauf Lawrence (1958) noch genauer eingeht, daß die Gartenformen ein recht komplexes Kreuzungsgemisch zwischen *Str. rexii*, *dunnii* und *insignis* darstellen. Nun aber geht hier in dieser Arbeit aus Tabelle 2 hervor, daß sich die gleiche Variabilität über sämtliche Antherenzustandsklassen hinweg in den Kreuzungsnachkommenschaften von *Str. wendlandii* × *baudertii* finden. Bei der Schilderung der experimentellen Ergebnisse ist schon *Str. baudertii* mit *Str. polyanthus* verglichen. Wir haben es also hier mit zwei verschiedenen reinen Formen zu tun, von denen die eine so gut wie überhaupt nicht variiert, *Str. polyanthus*, und die andere, *Str. baudertii*, eine enorme Streuung über den ganzen Bereich von Klasse 1—9 zeigt.

Daraus wird deutlich, daß die Variabilität in der Ausbildung des Staubblattkreises ebenfalls durch das Genom des väterlichen Elters bestimmt wird. Dabei scheint mir folgender Befund recht bemerkenswert zu sein. Wir hatten seinerzeit bei der Rückkreuzung Nr. 81, *Str. (wendlandii* × *baudertii)* × *baudertii*, darauf hingewiesen, daß man die ersten drei Klassen zusammenfassen kann und sie den letzten vier gegenüberstellen. Wir hätten demnach in den ersten drei bis vier Klassen die Variation des *wendlandii*-Genoms in seinem eigenen Plasmon bewertet. Demnach scheint es so zu sein, daß auch das plasmoneigene Genom durch den Einfluß des fremden Genoms zur Variabilität gebracht wird.

Welcher Art nun diese Variabilität eigentlich ist, kann man nach dem Bisherigen noch nicht genau sagen. Überblicken wir die Gesamtspaltung von *Str. baudertii*, so hat es durchaus den Anschein, als seien ebenso wie an der Variabilität der Blütenschlitzung mutative Prozesse daran beteiligt. Ob das der Fall ist, muß ebenso wie bei der Blütenschlitzung dadurch ermittelt werden, daß man Nachkommen von Blüten der gleichen Pflanze aufzieht. Darauf werden wir in einer späteren Arbeit noch einmal zurückkommen, und zwar im Zusammenhang mit den Kreuzungen zwischen *Str. wendlandii* und *cyaneus*. Die *cyaneus*-Kreuzungen haben, wie mir scheint, eine noch stärkere Variabilität, als die *baudertii*-Kreuzungen sie aufweisen.

Zusammenfassung der Ergebnisse

1. *Str. haygarthii* ist die einzige uns bekanntgewordene Art der Gattung *Streptocarpus*, die sowohl mit *Str. wendlandii* und *comptonii* als auch mit *Str. rexii*, *polyanthus* und *solenanthus* in beiden Richtungen gekreuzt, völlig normal fertile Bastarde in beiden Geschlechtern ergibt.

2. Alle sonstigen neu geprüften Arten lassen sich je nach der Intensität, mit der sie auf das Plasmon von *Str. wendlandii* reagieren, in einer Reihe einordnen mit *Str. gracilis* am Anfang, mit äußerst geringfügiger Reaktion auf das fremde Plasmon, bis zu *Str. montigena* mit maximaler. Dementsprechend entscheidet das plasmafremde Genom darüber, wie die Reaktion auf das gegensätzliche Plasmon erfolgt.

3. Vergleicht man die Tabelle 2 mit Tabelle 3 (Oehlkers 1957, S. 11), dann ergibt sich, daß die Blütenschlitzung an Intensität nicht mit derjenigen der Geschlechtsbestimmung übereinstimmt. Daraus läßt sich erneut schließen, daß beide Phänomene von verschiedenen Plasmonanteilen abhängig sind.

4. Die Variabilität der Geschlechtsausprägung ist ebenfalls durch das plasmonfremde Genom bestimmt. Sie ist maximal bei der Verwendung von *Str. baudertii*. minimal bei den *wendlandii-polyanthus*-Bastarden (vgl. Oehlkers 1945, S. 17). Dementsprechend wird auch die Variabilität des Phänotyps durch den plasmonfremden väterlichen Elter bestimmt.

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TEST OF A POSSIBLE CORRELATION BETWEEN CROSS-LINKING AND CHROMOSOME BREAKING ABILITIES OF CHEMICAL MUTAGENS

By

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With 1 Figure in the Text

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Almost without exception, alkylating agents with effective carcinostatic action possess two or more functional groups. From this it has been inferred that ability to cross-link biologically important macromolecules plays a major rôle in their action (Goldacre, Loveless and Ross 1949). In view of the renewed interest in this problem (Alexander, Cousens and Stacey 1957; Brookes and Lawley 1961) it seems worth while to report the results of experiments carried out several years ago, but so far referred to only briefly in another context (Auerbach 1960).

The rationale of these experiments was based on the following considerations. The ability of monofunctional compounds to produce mutations has been proved beyond doubt by a number of workers (Stevens et al. 1950); in some of these experiments monofunctional compounds were, in fact, better mutagens than similar bifunctional ones. Since, however, many or all of the mutations scored were point mutations, while the carcinostatic action of alkylating agents is more likely to be correlated with chromosome breakage, the possibility could not be excluded that monofunctional compounds are specifically deficient in chromosome-breaking ability. Tests with beta-propiolactone have shown long ago that at least this monofunctional compound can produce chromosome breaks in plants (Smith and Srb 1951). Whether quantitatively it is inferior in this respect to bifunctional compounds is not known and would, indeed, be difficult to assess without an independent measure of the amounts that effectively reach the genetic material. In *Drosophila*, in which sex-linked lethals can be used as a measure of effective penetration to the chromosomes, such a comparison between the chromosome-breaking ability of related monofunctional compounds can be carried out.

The substances chosen for comparison were ethylene oxide and diepoxybutane. Since these compounds are chemically as closely related as is possible for a monofunctional and a bifunctional one, they have already been compared in regard to other effects: sex-linked lethals in *Drosophila* (Bird 1952), reverse mutations in *Neurospora* (Kolmark and Westergaard 1953), chlorophyll mutations and sterility in barley (Ehrenberg and Gustafsson 1957). On a molar basis, ethylene oxide was found to be the weaker mutagen of the two for *Drosophila* and barley, but not for *Neurospora*. More relevant is the observation

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that after treatment of barley seeds with either compound a given frequency of chlorophyll mutations was associated with a higher frequency of sterile X 1 plants when diepoxybutane had been used. Since the Swedish workers consider inviable chromosome rearrangements to be the main cause of X 1 sterility, this finding would appear to support the idea that the monofunctional compound is a less efficient chromosome breaker than the bifunctional one.

In our experiments, genetically detected translocations involving thy Y and the two large autosomes were taken as measure of chromosome breakage, and the effective dose was estimated from the frequency of simultaneously induced sex-linked lethals. Expressed differently, the relative chromosome-breaking ability of the two compounds was expressed by the ratio of translocations to lethals; on the hypothesis that ethylene oxide is a markedly less efficient chromosome breaker than diepoxybutane, one would expect the translocation/lethal ratio to be higher after treatment with diepoxybutane than after treatment with ethylene oxide. This type of comparison can be used even for substances that, like the two used here, differ considerably in effectiveness on a molar basis.

Table. *Sex-linked lethals and translocations after treatment of adult ♂♂ with ethylene oxide (EO) or diepoxybutane (DEB)*

Agent	Molar conc.		Broods			
			1	2	3	4
EO	0.055	% l	1.18	1.65	3.21	0.89
		n	598	374	459	532
		tr	0	2	1	1
		% tr	0	0.53	0.22	0.19
		tr/l	—	0.32	0.07	0.21
EO	0.09	% l	5.32	3.70	3.38	3.34
		n	1074	539	653	727
		tr	3	4	3	1
		% tr	0.28	0.74	0.47	0.14
		tr/l	0.05	0.20	0.14	0.04
DEB	0.005	% l	6.52	7.43	8.95	4.03
		n	1151	1371	1371	1059
		tr	5	18	10	2
		% tr	0.43	1.39	0.73	0.18
		tr/l	0.07	0.19	0.09	0.04

%1 = percent sex-linked lethals; the values are based on 2—300 X-chromosomes in the first experiment, 600 to 1000 in the second, and 1100—1500 in the third.

n = number of spermatozoa tested for translocations.

tr = translocations between II, III, and Y.

Ethylene oxide (BDH) and diepoxybutane, both kindly supplied supplied by the Chester Beatty Institute for Cancer Research, were made up to different concentrations in 0.4% NaCl and injected into adult ♂♂ of a wild-type strain (OrK), which is habitually used for mutation experiments in our laboratory and has a spontaneous mutation frequency of 0.1—0.3% sex-linked lethals in ♂♂. On the day following treatment, the ♂♂ were mated to ♀♀ of the genotype *y sc^{S1} In 49 sc^{8}; bw;st*. Each ♂ was given two virgin ♀♀ every three days to produce four successive broods. Lethals were scored in the progeny of the daughters crossed to Muller-5 ♂♂, and translocations involving chromosomes II, III und Y were scored in the progeny of the sons mated to ♀♀ of the parental genotype. The results of two experiments with ethylene oxide and one with diepoxybutane are presented in the Table.

As in the experiments by Bird (1952), ethylene oxide produced fewer lethals per molar concentration unit than diepoxybutane. Even so, about 4000 spermatozoa from ♂♂ treated with ethylene oxide carried 15 translocations, so that the ability of this monofunctional compound to produce chromosome breaks in *Drosophila* cannot be doubted. For a comparison of relative translocation frequencies in the three experiments, the ratio of translocations to lethals (tr/l) has been calculated for each brood. As shown in the Table, these ratios are rather

similar in the corresponding broods of different experiments and certainly are not conspicuously higher after treatment with diepoxybutane. Since the brood patterns varied between experiments and series, probably because no care had been taken to standardize the collection of F_1 flies for testing, a brood-by-brood comparison between translocation/lethal rates seemed less useful than a comparison between averages, in which the vagaries of the brood patterns would be evened out. Such a comparison has been carried out graphically for the first three broods (Fig. 1). The fourth brood has not been included because it probably

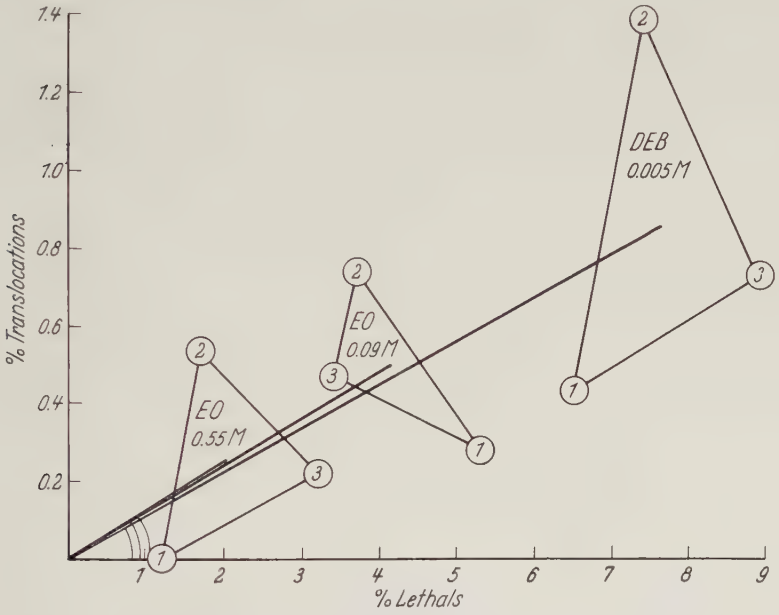

Fig. 1. Lethals and translocations in three successive broods (*1*, *2*, *3*) from DROSOPHILA ♂♂ injected with ethylene oxide (*EO*) or diepoxybutane (*DEB*)

contained a large proportion of progeny from meiotically or premeiotically treated germ cells. Even the third brood may have contained such cells, if sperm utilization in these experiments was similar to that in several other experiments with alkylating agents carried out by the same brood-technique (AUERBACH and SONBATI 1960, REDDI and AUERBACH 1961). However, after treatment with mustard gas, this rate of sperm utilization did not influence the translocation/lethal ratio in the third brood (SONBATI and AUERBACH 1960). Each triangle in Fig. 1 represents one experiment; the means of both lethal and translocation frequencies were determined by constructing the centre of gravity. When this point is connected with the centre of origin by a straight line, the angle between this line and the abscissa represents the mean lethal/translocation ratio for the three broods. It will be seen that the three angles are very nearly the same. Expressed differently, the ratio of translocations to lethals was very similar in all experiments and for both substances.

Taken at its face value, this seems clear proof that, measured in terms of mutagenically effective amounts, ethylene oxide is as effective as diepoxybutane

in breaking the chromosomes of *Drosophila*. This conclusion, however, can be accepted only with certain reservations. A surprising feature of these experiments was that translocation frequency after treatment with diepoxybutane was, in fact, lower than might have been expected on the hypothesis that both substances are equally effective chromosome-breakers. It has been shown for mustard gas (Nasrat et al. 1954) that the frequency of translocations increases as the square of the frequency of sex-linked lethals, indicating that mustard gas, like X-rays, produces translocations from two independently induced chromosome breaks. For another polyfunctional alkylating agent, triethylene melamine, a similar conclusion can be drawn from dose-effect curves presented by Fahmy and Bird (1953). If the same should apply to diepoxybutane, our data would suggest that, at lethal frequencies as low as those produced in our experiments with ethylene oxide, diepoxybutane would produce noticeably *fewer* translocations than ethylene oxide. This might, e.g., be so if breaks produced by diepoxybutane had a lower rejoining ability than breaks produced by ethylene oxide. Alternatively, the dose-effect curve for translocations after treatment with diepoxybutane might be linear rather than quadratic. Both possibilities are now under test.

A source of error which may have contributed to the discrepancy arises from the possibility that the lethals on which the dose-estimates were based may have contained different proportions of large rearrangements. If the square dose-effect law can be applied, this would result in a relative over-estimate of dose for the substance with the higher proportion of rearrangements among the lethals. Unfortunately, these experiments were carried out several years ago, and the lethals are no longer available for cytological analysis. Fahmy and Bird (1953) found about 10% large rearrangements among sex-linked lethals that had been induced by a dose of diepoxybutane yielding an overall frequency of 10% lethals. If this value can be applied to our experiment, the maximal over-estimate of dose is too small to account for the observed shortage of diepoxybutane-induced translocations. Experiments now under way with ethylene imine as the monofunctional and triethylene melamine as the polyfunctional compound will be combined with cytological checks for the proportion of large rearrangements among the lethals.

A recent investigation by Fahmy and Fahmy (1961) led them to the conclusion that, within the group of mesyloxy esters, monofunctional compounds are less effective than bifunctional ones in the production of chromosome breaks leading to major rearrangements. However, since their bifunctional compound was a much weaker mutagen than the monofunctional ones and produced no translocations at all in over 2000 tested spermatozoa (although some were found among cytologically analysed lethals from a different experiment with the highest tolerated dose), no comparison with our results is possible.

Summary

The frequencies of sex-linked lethals and translocations were measured in the progeny of *Drosophila* ♂♂ injected with either ethylene oxide or diepoxybutane. The ratio of translocations to lethals was the same after either type of treatment. This lends no support to the idea that the difference in carcinostatic activity between monofunctional and polyfunctional alklating agents is

due to the cross-linking action of the latter type of compound on the chromosomes. Diepoxybutane did, in fact, induce *fewer* translocations than expected on the square dose-effect law for large rearrangements. Possible causes for this finding are discussed.

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Kurze Mitteilung

Aus dem Max-Planck-Institut für Biologie, Abt. Melchers, Tübingen

DIE AUFHEBUNG EINES DEFEKTES DER ZELLTRENNUNGEN BEIM *SNAKY*-STAMM VON *PARAMAECIUM AURELIA* IN SALZLÖSUNGEN

Von

ROLAND MALY

(Eingegangen am 15. August 1961)

Bei der Mutante *snaky* von *Paramaecium aurelia* var. *4* bleiben bei sonst normalem Zellwachstum und normaler Kernteilung durch eine Hemmung der Zelltrennung mehrere Tochtertiere ketten- oder klumpenförmig miteinander verbunden. Die Hemmung der Zelltrennung ist in ihrem Ausmaß variabel und stark durch Milieufaktoren zu beeinflussen. Die für den *snaky*-Charakter monohybrid spaltende, rein karyotisch bedingte Mutante konnte phänotypisch durch Kultur in sauerstoffarmem Medium oder durch Zusatz von Kohlenmonoxyd (nur im Dunkeln wirksam) oder Fe^{++}- oder Co^{++}-Salzen zum Kulturmedium, in dem *Aerobacter aerogenes* als Futterorganismus vorhanden ist, völlig normalisiert werden: die Zelltrennungen verliefen ebenso störungsfrei wie beim Normalstamm und es entstehen weder Ketten noch Klumpen. Umgekehrt wurde die Störung der Zelltrennung verstärkt in sauerstoffreichem Milieu oder durch Zusätze von Monojodessigsäure oder Äthylendiamintetraessigsäure (EDTA) zum Medium. Auf Grund der gegensätzlichen Wirkung von EDTA und Eisen (oder dem gleich wirksamen Kobalt) wurde vermutet, daß bei *snaky* im Gegensatz zum Normalstamm die Funktion der Aldolase unter weitgehend aeroben Bedingungen gestört sei, und daß ein Zusammenhang zwischen den für die Zelltrennung nötigen Prozessen und dem Glykolysestoffwechsel anzunehmen ist (MALY 1960a, b).

In den zitierten Arbeiten war die Versuchsanordnung so getroffen, daß mit den gewählten Bedingungen (Sauerstoffmangel, Kohlenmonoxyd- oder Eisen- oder Kobalt-Zusatz), die während des Wachstums und der vegetativen Vermehrung wirksam waren, das Auftreten der Störungen der Zelltrennung verhindert wurde. Es war nun die Frage zu prüfen, ob eine schon bestehende Hemmung der Zelltrennung so zu beheben ist, daß sich zusammenhängende Tiere voneinander trennen können, und zwar möglichst unter solchen Bedingungen, die mangels Futterorganismen *(Aerobacter)* kein weiteres Wachstum zulassen. Ferner schien es verlockend, die Annahme, daß bei *snaky* die Aldolase gestört ist, mit einer Abwandlung des klassischen Verfahrens, das BEADLE und TATUM erstmals an *Neurospora* anwandten, zu prüfen. Wenn nämlich die Ursache für die Störung der Zelltrennung in einer Hemmung der Aldolasereaktion liegt, so müßten die Schwierigkeiten behoben werden können, wenn den Tieren ein Reaktionsprodukt des blockierten Enzyms angeboten wird; im Falle der Aldolase, die Fructosediphosphat zu Phosphoglycerinaldehyd und Dihydroxyacetonphosphat spaltet, also die Triosephosphate. Fructosediphosphat hingegen müßte ohne Wirkung bleiben.

Es wurden zu diesen Versuchen aus hungernden Kulturen, in denen keine Vermehrung der Paramaecien mehr stattfand und die von Bakterien „klargefressen" waren, kleine Monstra, die aus zwei Einheiten bestanden, mit einer Mikropipette einzelnen in je etwa 0,5 ml Salzlösung isoliert. Die Salzlösung sollte ein weiteres Wachstum der Paramaecien verhindern und mitgeschleppten Bakterien keine Möglichkeit zu rapider Vermehrung geben. Die Festigkeit der Verbindung zwischen den zusammenhängenden Individuen wurde durch mehrmaliges, kräftiges Auspressen mit der Pipette geprüft.

Die Salzlösung bestand aus (0,04% NaCl, 0,01% KCl, 0,02% $CaCl_2$, 0,01% $MgSO_4$, 0,005 K_2HPO_4, 0,005 KH_2PO_4; p_H 6,5).

Salzlösung ohne Zusätze diente als Kontrolle und war stets im gleichen Umfang angesetzt wie die „Versuchsreihe" mit TPE-, Fe^{++}-, oder Co^{++}-Zusatz. Temperatur 27° C, Dunkelheit.

Durch Zusatz der Triosephosphorsäureester (TPE als Na-Salze, Boehringer) wurde der p_H mehr nach der sauren Seite (p_H 5,7) verschoben. Um sicherzustellen, daß die beobachteten Effekte nicht durch die p_H-Verschiebung mit beeinflußt sind, wurde nach den ersten Versuchsserien (s. Tabelle 1 „ohne Puffer"), in denen niemals eine Vermehrung der Tiere festgestellt werden konnte, eine stärkere Pufferung versucht. Bei Verwendung von Phosphatpuffer (m/100) in der Salzlösung vermochte weder TPE noch Eisensulfat, die sich vorher wirksam erwiesen hatten, die Durchschnürung der Tiere zu beeinflussen. Sehr geeignet war dagegen Mc-Ilvaine-Puffer (Zitronensäure/Phosphat) und gleichermaßen Citrat/NaOH-Puffer (1:10 verdünnt). Der einzige Nachteil der citrathaltigen Puffer ist, daß ein geringfügiges Wachstum der Tiere innerhalb der Versuchszeit festzustellen war.

17—18 Std nach Versuchsansatz wurde ausgezählt, wie viele Trennungen erfolgt waren. Die Differenz der Häufigkeit von Trennungen zwischen der jeweiligen Versuchsreihe und der in gleichem Umfang mitlaufenden Kontrolle ist das eigentliche Versuchsergebnis. In der Tabelle ist neben dem Mittelwert der Differenzen zwischen Kontrolle und „Versuch" ($\bar{x}$) noch die Anzahl der Versuchsansätze (mit je 60 oder 90 „2-er-Monstra" in Kontrolle und Versuch) (N) und schließlich noch der mittels des t-Testes errechnete P-Wert zur Prüfung der Nullhypothese angegeben.

Tabelle (Erläuterung s. Text)

Versuch	$\bar{x}$	N	P
TPE[1] (ohne Puffer)	31,0	23	$< 0{,}001$
TPE[1] (McIlvaine-Puffer) .	29,7	13	$< 0{,}001$
TPE[1] (Citrat/NaOH-Puffer)	16,0	3	0,05—0,02
Fe^{++}[2] (ohne Puffer)	40,6	8	0,01—0,001
Fe^{++}[2] (McIlvaine-Puffer) .	17,0	8	$< 0{,}001$
Co^{++}[3] (McIlvaine-Puffer) .	12,8	4	0,05—0,02

[1] Konzentration $4 \cdot 10^{-6}$ mol.

[2] Konzentration 10^{-4} mol.

[3] Konzentration $5 \cdot 10^{-6}$ mol $\cdot Co(NO_3)_2$ und $CoCl_2$ waren in gleicher Weise wirksam.

Aus der Tabelle ist klar zu ersehen, daß Zusatz von TPE-, Ferrosulfat und Cobalto-Salzen die Vollendung der gestörten („steckengebliebenen") Trennung der Zellen (Zytokinese) in statistisch signifikanter Weise fördert, und zwar ohne gleichzeitig stattfindendes Zellwachstum. Die strengste Aussagekraft kommt den Versuchen mit $FeSO^4$-Zusatz zu reiner Salzlösung ohne Puffer zu, denn eine Vermehrung mitgeschleppter Bakterien in reiner Salzlösung mit Eisenzusatz ist undenkbar.

Fructosediphosphat wurde in 21 Versuchsansätzen ohne den geringsten Erfolg der Salzlösung zugegeben. Ebenso negativ sind 8 Versuchsansätze mit Adenosintriphosphat und 3 Versuchsreihen mit Zusatz von Glukose-6-phosphat geblieben.

Die positiven Resultate mit TPE und die Unwirksamkeit von Fructosediphosphat bei der gegebenen Versuchsanordnung bilden eine weitere Stütze für die Annahme einer Beziehung zwischen der Aldolasefunktion und den Vorgängen bei der Zytokinese.

Summary

From the *snaky* strain of *Paramaecium aurelia* small monsters consisting of two non separated units were isolated into an inorganic salt solution. A significant higher percentage of cell separation was effected by the addition of triose phosphates (see Table TPE), but not by adding fructose 1,6-diphosphate. A better separation was also obtained by adding divalent iron ($FeSO_4$) or cobalt salts. The results give further support to the hypothesis, that the defect of cell separation is connected with the function of aldolase.

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